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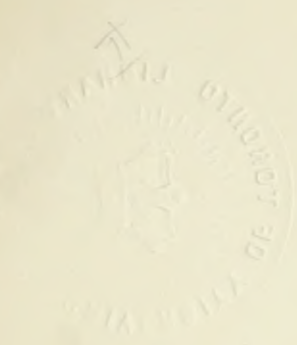
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VARIATIONS IN THE VIRULENCE OF DIFFERENT STRAINS OF *BACILLUS DIPHThERIAE*.

By J. A. ARKWRIGHT, M.D.

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THE occurrence of non-virulent strains of bacilli, which are indistinguishable except by animal experiment from virulent diphtheria bacilli, makes it especially interesting to investigate the varying pathogenicity of this bacillus. Owing to their complete lack of pathogenicity for laboratory animals, the exact relationship of the non-virulent types of *B. diphtheriae* to the virulent forms of this organism, cannot yet be considered as settled, although Arkwright (1910) has succeeded in obtaining a slight but very definite development of antitoxin in the blood of a horse immunised with non-virulent strains.

In this paper no cognisance will be taken of the non-virulent forms. The experiments here recorded deal solely with the variations in virulence which are found to exist among strains which exhibit a definite pathogenicity for guinea-pigs.

The pathogenicity or virulence in a general sense has been examined by means of "whole" broth cultures containing both bacilli and the toxin formed by them, but an attempt has also been made to determine whether such differences in lethal power as occur between different strains are due chiefly to differences in the amount of toxin in the cultures, or to differences in the virulence proper of the bacillus, *i.e.* the activity of the bacillus after inoculation in the toxin-free state into the animal body.

Difference in the Pathogenicity of different strains.

Many writers on this subject have recorded great variations in the pathogenicity of different strains, *e.g.* v. Behring (1901), Bardach (1895), Martin, L. (1898), Pennington (1907), and the matter is thoroughly

discussed by Graham-Smith and Dean in *The Bacteriology of Diphtheria*, (Nuttall and Graham-Smith (1908)).

Graham-Smith (1904) and Cobbett (1901), on the other hand, found a remarkable uniformity in the lethal dose of the strains which they isolated during two large epidemics of diphtheria at Cambridge. Cobbett out of 68 virulent strains, found only two which when recently isolated did not kill a guinea-pig of 250 grms. in two or three days, when 0.1 c.c. of a two-day broth culture was injected subcutaneously. (In four instances only, 0.5 c.c. was the smallest dose used.) The two strains of exceptionally low virulence were rapidly raised to the same degree of pathogenicity as the remainder, after one or more subcultures in broth.

Graham-Smith amongst 88 virulent strains isolated, found one which did not kill within two or three days with the smallest dose used. The doses injected varied from 0.1 to 0.3 c.c. but the majority of strains killed in a dose of not more than 0.2 c.c. With only one strain, 0.1 c.c. did not kill the experimental animal in 12 days, but the growth was very poor and subculture in broth did not materially increase its vigour.

It is to be noted, however, that these writers do not record any observations as to variations in virulence above these limits, *i.e.* when smaller doses than 0.1 c.c. were used. Moreover all the strains were isolated during two epidemics in the same town and their work shows the high degree of virulence of *B. diphtheriae* during these two epidemics.

Williams (1902) also noted the occurrence of strains of low virulence which attained a higher grade of virulence when the growth on broth had been improved by subculture.

Smith, Th. and Walker, E. (1896) tested the virulence of different strains by estimating the accumulation of toxin in broth cultures, and came to the conclusion that the virulence of different strains was very uniform. They did not, however, test the pathogenicity of cultures containing bacilli, and their conclusions are remarkable when one considers the enormous differences in the toxin-yield from broth cultures which have been obtained with different, or even the same, strains by many workers, *e.g.* Park (1896), Dean (1908), Madsen (1907).

I have not infrequently met with strains of low pathogenicity. In a school epidemic which I investigated (Arkwright, 1908), of 13 virulent strains which were isolated, only four were of such a degree of virulence that 0.1 c.c. of a two-day broth culture killed a guinea-pig of

250 grms. within four days and 2 c.c. in 48 hours. Three other strains when given in a dose of 0.1 c.c. killed the animal in ten days whereas a dose of 2 c.c. was fatal within four days. The remaining six strains were non-lethal when 0.1 c.c. was injected, but a dose of 2 c.c. was fatal within four and a half days.

At that time it seemed possible that this prevalence of strains of low virulence, might be causally connected with the large number of completely non-virulent strains found in the same epidemic (seven strains were non-virulent out of 20 strains which were isolated), and it seemed not unlikely that different degrees of virulence might prevail in different epidemics.

In the case of a small epidemic in a school from which I have recently examined material, five cases of diphtheria occurred. From these patients three strains of *B. diphtheriae* were isolated and tested for virulence. All were found to be of low virulence, when tested on several occasions. Although a large dose of 2 c.c. or 2.5 c.c. killed a guinea-pig rapidly, and a control animal to which antitoxin was given was unaffected, yet 0.1 c.c. proved to be a sublethal dose.

In another series of 37 virulent strains from various sources 25 were lethal when 0.1 c.c. and the remainder when 2.5 c.c. were injected.

Method of estimating pathogenicity.

In estimating the pathogenicity¹ of a strain of *B. diphtheriae* by the injection of "whole" two-day cultures (bacilli and toxin together) several difficulties were met with.

No doubt the exact composition of the broth in which the bacilli are grown makes a considerable difference in the results obtained by injecting whole cultures, and occasionally strains which are generally of low pathogenicity attain a much higher grade when grown on a different batch of broth, but when this happens there is sometimes reason to believe that the strains of high pathogenicity in cultures made at the same time, and in the same broth, are correspondingly exalted. Graham-Smith and Cobbett attach great importance to the use of sugar-free broth, but other workers have found that so long as the broth culture remains alkaline in reaction, the presence of a small amount of sugar does not hinder the formation of toxin (*e.g.* Smith, Th., Park and Williams).

¹ In this paper when referring to my own work the term "Pathogenicity" is used when "whole" broth cultures, "Toxigenicity" when toxin free from bacilli, and "Virulence" when washed bacilli, have been employed for injection.

In this investigation the cultures in broth were found to remain alkaline. The broth employed was made from bullock's heart with 1 % of Witte's peptone added, and rendered just alkaline to litmus paper. That the variations in the broth used were not an important cause of the differences in the results with the different strains, was shown by the fact that all degrees of virulence were found with the same brew of broth.

The period of incubation was two days at 37° C.

Estimation of the amount of growth in broth.

The growth in broth is obviously not always equally great when the same strain is used, and with different strains the amount and character of the growth varies very much.

Williams, Cobbett and Graham-Smith attribute the low virulence of their exceptional strains to the scanty growth in broth when they were recently isolated. In the case of my strains of low pathogenicity the growth was usually, but not always, small, and was not less than that of some strains possessing a higher lethal power. Training the bacilli to grow in peptone broth will sometimes increase the pathogenicity somewhat, but it is possible that other changes than increased ability to grow on artificial media may be induced in this way.

In order to estimate the amount of growth 10 c.c. of the bouillon cultures were centrifuged in a tube drawn out to a small calibre in its lower half and graduated in hundredths of a c.c. in this part. The amount of growth as shown by the deposit measured in this way was not proportionate to the virulence.

It was not found possible to count the bacilli in a broth culture on account of the clumping of most strains.

The following strains were used :

Strain No. I.	11 Bb	Isolated 24 Jan. 1911 from a diphtheria convalescent.
II.	66	„ 2 Feb. 1911 from a school "carrier."
III.	134 X	„ 21 Oct. 1909. Diagnosis swab.
IV.	134 Z	„ 21 Oct. 1909. „ „
V.	19 AT	„ 25 Feb. 1911 from a diphtheria convalescent.
VI.	21 AN	„ 25 Feb. 1911 „ „ „
VII.	R 50	„ 6 Dec. 1910. Case of diphtheria.
VIII.	R 4	„ 7 Dec. 1910. „ „
IX.	166	Skin infection by <i>B. diphtheriae</i> .
X.	27	Isolated 14 Mar. 1911. Diphtheria convalescent.
XI.	Dkth	„ 12 Jan. 1911. „ „
XII.	NE 30	„ 16 Mar. 1911. „ „
XIII.	R 70	„ 31 Mar. 1911. Case of diphtheria.

The majority of the strains were chosen on account of their low virulence, but the extremes of virulence met with are represented. Some of the strains have been examined at considerable intervals of time with the object of ascertaining whether the degree of virulence remained fairly constant.

The results of injection of the "whole" two-day cultures are shown in Table I.

In this table the dose stated to be the M.L.D. is that of a two-day broth culture which caused death within four days. In another column is shown the sub-lethal dose, *i.e.* the highest dose which allowed the animal to survive. The approximate amount of deposit from 10 c.c. of the culture is also shown. The guinea-pigs were all between 240 and 250 grms. in weight. The doses used were usually 0·01, 0·02, 0·1, 0·5 and 2·5 c.c.

In Table II are recorded a few of the observations in which several strains of different degrees of virulence were tested on the same day in the same broth, showing that large variations in virulence were obtained with the same quality of broth.

In considering the results shown in Table I, it is seen that of the seven strains, I, II, IX, XI, V, VI, VII, each of which was examined on several occasions, four, *viz.* I, II, IX and V, remained fairly constant in pathogenicity. Of these I and II were of high pathogenicity, having a very small M.L.D. varying at most from 0·01 to 0·02 c.c. in the case of strain I, and from 0·02 to 0·1 c.c. in the case of strain II. The other two constant strains IX and V were of low pathogenicity, the M.L.D. varying from 0·5 to 2·5 c.c. Of the three remaining strains (XI, VI and VII) which were examined on more than two occasions, the M.L.D. varied from 2·5 to 0·1 c.c. Strain X was tested on only one occasion. Strain VIII was tested twice and on both occasions was of low pathogenicity.

The amount of deposit obtained by centrifuging 10 c.c. of culture varied for different strains between 0·0025 and 0·02 (1 to 8), but the amount of deposit was not proportionate to the pathogenicity of the culture, for the M.L.D. of culture for these strains varied from 2·5 to 0·01 (250 to 1). Thus strains I and II gave deposits only varying from 0·005 to 0·02 c.c. (1 to 4) and the M.L.D. for the same two strains varied from 0·1 to 0·01 c.c. (100 to 1).

In the case of strain IX the amount of deposit varied from 0·0025 to 0·005 c.c. with a M.L.D. never less than 0·5 (tested on four occasions). In the same way strain V gave a deposit of 0·0025 to 0·0075 c.c. but the M.L.D. was never less than 0·5 c.c.

TABLE I.

Strain and date of isolation	Dates of experiments	Deposit from 10 c.c. of culture	M. L. D. of "whole" culture	Day of death	Sublethal dose	Result of do.
No. I	4 Feb. 1911	—	0·1	Second	—	—
24 Jan. 1911	11 Feb. „	—	0·02	Third	—	—
	16 Feb. „	0·015	0·01	Third	—	—
	15 May „	0·005	0·02	Second	—	—
No. II	9 Dec. 1909	—	0·1	—	—	—
2 Feb. 1909	3 Feb. 1911	—	0·1	Third	0·02	Large swelling.
	9 Mar. „	0·01	—	—	—	—
	15 May „	0·02	0·02	Third	—	—
No. IX	13 Jan. 1911	—	2·5	Second	0·1	Almost nil.
11 Jan. 1911	22 Feb. „	—	—	—	1·5	Death 5th day.
	24 Feb. „	—	0·5	Third	0·25	Death 10th day.
	9 Mar. „	0·0025	—	—	—	—
	6 Apr. „	—	0·5	Second	0·1	Death 5th day.
	20 Apr. „	0·005	—	—	—	—
	28 Apr. „	0·0025	0·5	Second	0·1	Death 8th day.
No. X	20 Mar. 1911	—	0·1	Second	—	—
14 Mar. 1911	27 Mar. „	0·003	—	—	—	—
No. XI	18 Jan. 1911	—	2·5	Third	0·1	Lived.
12 Jan. 1911	6 Apr. „	—	0·5	Fourth	0·1	Death 7th day.
	28 Apr. „	0·003	0·5	Second	0·1	Lived.
	15 May „	0·003	0·1	Third	—	—
No. V	6 Apr. 1911	—	0·5	Second	0·1	Lived.
25 Feb. 1911	20 Apr. „	0·0075	2·5	Fourth	0·5	Lived.
	28 Apr. „	0·0025	0·5	Second	0·1	Death 5th day.
No. VI	11 Mar. 1911	—	2·5	Second	0·1	Lived.
25 Feb. 1911	6 Apr. „	—	0·1	Fourth	—	—
	20 Apr. „	0·005	—	—	—	—
	28 Apr. „	0·0025	0·1	Second	—	—
No. VII	10 Dec. 1910	—	2·5	Second	0·1	Lived.
7 Dec. 1910	14 Dec. „	—	—	—	2·0	Lived.
	5 Apr. 1911	—	2·5	Second	0·5	Lived.
	21 Apr. „	0·005	—	—	—	—
	15 May „	0·003	0·1	Third	—	—
	15 May „	—	0·5	Fourth	—	—
No. VIII	12 Dec. 1910	—	2·5	Second	0·1	Lived.
7 Dec. 1910	5 Apr. 1911	—	2·5	Second	0·5	Death 5th day.
	21 Apr. „	0·005	—	—	—	—

TABLE II.

Observations on different strains on the same date showing that the broth used was not the cause of differences in virulence.

A.

Strain	Date of experiment	Deposit from 10 c.c. of culture	M.L.D. whole culture	Day of death	Deposit from 10 c.c. of emulsion	M.L.D. original emulsion of bacilli	Day of death	Sublethal dose original emulsion	M.L.D. standard emulsion	Day of death	Sublethal dose standard emulsion
No. I	15 May '11	0.005	0.02	Second	0.009	0.02	Second	—	0.02	Second	—
No. II	"	0.02	0.02	Third	0.01	0.1	Third	0.02	0.1	Third	0.02
No. XI.	"	0.003	0.1	Third	0.015	0.33	Third	0.066	0.5	Third	0.1
No. VII.	"	0.003	0.1	Third	0.003	1.5	Third	0.3	0.5	Third	0.1

B.

Strain	Date of experiment	M.L.D. whole culture	Day of death	Sublethal dose
No. VII	5 Apr. 1911	2.5	Second	0.5
No. XII	"	0.5	Fourth	0.1
No. XIII	"	0.1	Second	—
No. VIII	"	2.5	Second	0.5

C.

Strain	Date of experiment	M.L.D. standard emulsion	Day of death	Sublethal dose
No. I	6 Mar. 1911	0.01	Second	—
No. XI	"	4.0	Third	1.0

The variation in deposit for the same strain was not proportionate to the changes in pathogenicity, *e.g.* strain V on the occasion on which the deposit was largest (0.0075 c.c.) had a M.L.D. of 2.5 c.c., whereas on another occasion with a smaller deposit of 0.0025 to 0.003 c.c. the M.L.D. was actually smaller, *viz.* 0.5 c.c.

Strain XI twice gave a deposit of 0.003 c.c. but on one occasion the M.L.D. was 0.5 c.c. and on the other 0.1 c.c.

It appears, therefore, that neither in regard to different strains, nor to different observations on the same strain, were the comparatively small variations in the amount of deposit proportional to the pathogenicity of the culture.

The difference in pathogenicity between the strains was much greater than could be accounted for by the difference in the amount of growth in the respective cultures.

Although the degree of pathogenicity of the same strain varied from time to time, the difference between the strains for the most part remained well marked.

*The independent variation of Bacilli and Toxin as regards
Lethal power in two-day broth cultures.*

Workers who have investigated the relation of virulence to toxigenicity of different strains of *B. diphtheriae* have usually made use of 24-hour broth or young agar cultures in estimating the virulence, and filtrates from broth cultures of about a week old, for testing the amount of toxin produced. They have come to the conclusion that the same bacillus may be highly virulent and yet of feeble toxigenic power or the reverse (Martin, L., 1898, Behring, 1901). In the tests recorded in the present communication the virulence and toxigenicity have both been examined by means of a two-day broth culture.

It seemed that from the point of view of virulence it was of more interest to know the relation, as regards lethal power, between the toxin and bacilli in the same culture. Moreover it seemed unlikely that the amount of toxin accumulated in a 7-day broth culture had any very intimate relation to the pathogenicity of a particular strain, as the experimental animal injected with bacilli or culture usually dies within three days. Also the exact composition of the broth probably has a special effect on the accumulation of toxin independently of its formation.

Methods of testing the Toxigenicity and Virulence.

A measured quantity of the culture was centrifuged and the clear fluid poured off and recentrifuged. The resulting clear fluid was tested for its toxin-content.

The deposit after the first centrifuging was washed with salt solution, and after again centrifuging the deposit was made up to the original volume. The M.L.D. of the resulting bacterial emulsion was taken as a criterion of "Virulence proper."

The doses of toxin or bacterial emulsion employed were 0.01, 0.02, 0.1, 0.5 and 2.5 c.c. and occasionally doses with smaller intervals were used.

In order to reduce the dose of bacilli to a uniform measure a standard bacillary emulsion was used of such a strength that 10 c.c. of emulsion, on centrifuging, yielded a deposit of 0.01 c.c. of bacilli. The M.L.D. of bacilli could then be stated as a dose of such a standard emulsion. In a few instances a standard emulsion was actually made, and used for

Table III shows that the M.L.D. of standard emulsion varied for the same strain at most from 0·1 to 2·0 c.c. in the case of No. II, but so large a M.L.D. as 2 c.c. was quite unusual for this strain. Leaving this one observation out of consideration the greatest variation for a single strain was in the case of No. XI which on different occasions gave a M.L.D. of 4 c.c. and 0·5 c.c. (variation of 8 to 1). The differences amongst the different strains, if one takes the occasions of least difference, ranged from 0·5 to 0·01 (50 to 1), or if one takes the greatest differences, from 4 to 0·01 (400 to 1).

The nine strains fall roughly into three groups as regards their virulence as tested by the M.L.D. of standard emulsion.

- (1) Two strains, I and X, had a M.L.D. of 0·01 to 0·02 c.c.
- (2) Three strains, IX, V and VI, had a M.L.D. of 0·1 to 0·17 c.c.
- (3) Three strains, XI, VII and VIII, whose M.L.D. was 0·5 c.c. or more.

Strain II, whose M.L.D. varied from 2·0 to 0·1 c.c., should probably be included in the second group; this strain had been tested for pathogenicity on several previous occasions and the M.L.D. was never more than 0·1 c.c. The occasion on which the M.L.D. of original emulsion and standard emulsion was 2·0 c.c. was altogether exceptional.

The M.L.D. of standard emulsion and of original emulsion of the same strain were not widely different when they were both examined on the same occasion. This is merely another way of stating that the differences in deposit yielded by the original emulsions of the different strains were not very large, and further that variations in the inherent properties of the bacillus were of more importance when determining the M.L.D. than variations in the number of bacilli present.

There is no evidence to show whether the greater virulence of some strains is due to increased resistance to the destructive powers of the animal body, greater rapidity of multiplication in the body or a greater ability to make toxin from the body fluids.

In Table IV the M.L.D. of toxin and that of the emulsion of washed bacilli made up to the original bulk of culture after centrifugalising, are shown together. The ratios of the minimal lethal doses of original emulsion and of the standard emulsion to the M.L.D. of toxin occurring in the same culture, are given in order to show the relative values of the bacilli and toxin as lethal agents in two-day cultures.

The ratio of the M.L.D. of the bacilli to that of the toxin obtained from the same culture was most commonly as 1/1; this was the case for 7 out of 11 strains examined, and shows that the lethal power of the

toxin and bacilli in a two-day culture are usually equal. This relation was curiously maintained, even when the same strain yielded cultures of high and low pathogenicity on different occasions, as in the case of strain VI.

TABLE IV.

Comparison of the lethal power of washed bacilli and toxin in the same cultures (two days' growth).

Strain	Date of experiment	M.L.D. of original emulsion	M.L.D. of standard emulsion	M.L.D. of toxin	Ratio of M.L.D. of original emulsion to M.L.D. of toxin	Ratio of M.L.D. of standard emulsion to M.L.D. of toxin
No. I	11 Feb. 1911	0·01	0·01	0·5	1/50	1/50
„ II	3 Feb. „	0·1	—	0·1	1/1	—
„ „	9 Mar. „	2·0	2·0	0·5	1/0·25	1/0·25
„ III	21 Dec. 1909	0·1	—	0·1	1/1	—
„ IV	21 Dec. „	0·1	—	0·1	1/1	—
„ V	20 Apr. 1911	0·5	0·17	2·5	1/5	1/15
„ VI	16 Mar. „	4·0	—	4·0	1/1	1/3
„ „	20 Apr. „	0·5	0·17	0·5	1/1	1/3
„ VII	21 Apr. „	> 2·5	> 0·83	2·5	1/1	1/3
„ VIII	21 Apr. „	2·5	1·25	2·5	1/1	1/2
„ IX	9 Mar. „	0·1	—	1·0	1/10	—
„ „	20 Apr. „	0·5	—	2·5	1/5	—
„ X	27 Mar. „	0·05	0·02	1·0	1/20	1/50
„ XI	10 Feb. „	> 3·0	4·0	3·0	1/1	1/0·75
„ „	20 April „	2·5	—	2·5	1/1	—

On the other hand the M.L.D. of bacillary emulsion of strains I, V, IX and X was smaller than that of the toxin, the ratio $\frac{\text{M.L.D. bacilli}}{\text{M.L.D. toxin}}$ being represented by the fractions $\frac{1}{50}$, $\frac{1}{3}$, $\frac{1}{10}$ or $\frac{1}{3}$ and $\frac{1}{20}$ respectively, so that, *e.g.* in the case of strain I, the bacilli in the culture had actually fifty times the lethal power of the toxin.

When the M.L.D. of standard emulsion is compared in the same way with the M.L.D. of toxin, none of the strains showed an equal value of standard emulsion and toxin. In two cases, *e.g.* strains II and XI, the M.L.D. of standard emulsion of bacilli, was actually slightly greater than that of the toxin, but more commonly the M.L.D. of toxin was the greater. For example in the cases of strains VI, VII and VIII the ratio $\frac{\text{M.L.D. of standard emulsion}}{\text{M.L.D. of toxin}}$ was equal to $\frac{1}{3}$, $\frac{1}{3}$ and $\frac{1}{2}$ respectively, and in strains I and X the ratio was represented by $\frac{1}{50}$ in each case.

This method of comparison emphasises the fact that in a two-day culture the lethal value of the bacilli equals or may greatly exceed that

of the toxin, and that variation in the pathogenicity of the whole culture depends on the bacilli more than on the toxin.

Some strains, therefore, were apparently far better producers of toxin in the body than in vitro, perhaps on account of their greater resistance to the adverse influences met with in the body or on account of their more rapid multiplication there.

This is perhaps as good a criterion as can be found of their virulence proper, and such strains may be correctly described as of higher virulence than strains in which the toxin production in vitro and in vivo are more equal.

Irregularities.

The Minimal Lethal Dose of "whole culture," of toxin or of bacillary emulsion was not constant for individual strains, and this was not to be expected. There was, however, a very fair amount of agreement for most of the strains on the different occasions on which they were tested. Four strains of low virulence (Nos. V, VI, VII and XI) were retested after four passages through broth and in only one case (No. VI) did a smaller dose of the whole culture prove fatal.

The strains of low virulence which have been observed are widely separated from completely non-virulent strains. The difference may not, however, be much greater than that between strains of very high and very low virulence. The relation of the M.L.D. of a strain of very high virulence to one of low virulence may be expressed as a ratio of 1 to 50 or more. If the M.L.D. of a strain of low virulence bore the same ratio to the M.L.D. of a "non-virulent" strain, the latter would be lethal in a dose of 50 c.c. whereas a dose larger than 10 c.c. cannot be conveniently used.

Incidental observations.

In order to find out whether any considerable part of the lethal effect of injecting washed bacilli was due to toxin clinging to the bacilli, toluol (on other occasions chloroform) was added to the emulsion of washed bacilli and also to the separated toxin. They were then kept at room temperature for 24 to 48 hours till all the bacilli in the emulsion were dead. The M.L.D. of toxin was then found not to have altered materially but the bacilli had become almost innocuous, and 30 to 40 times the dose of bacillary emulsion, which when living caused death, now caused hardly any disturbance. With a similar object, a small

amount of antitoxin was added to the bacillary emulsion and the mixture incubated for half an hour and then centrifuged, the bacilli again washed and the emulsion made up to the original volume. The M.L.D. of bacilli remained about the same as that of the emulsion before the antitoxin was added.

It was thought that possibly strains of low virulence, or strains with no pathogenic action, in reality formed toxin but had acquired the property of destroying it with greater rapidity than ordinary virulent strains. Two-day cultures of non-virulent bacilli and virulent bacilli grown together were, however, almost as lethal as pure cultures of virulent bacilli.

CONCLUSIONS.

(1) The pathogenicity of different strains of *B. diphtheriae* when first isolated, as tested with two-day broth cultures, varies greatly (Minimal Lethal Dose varies as 400 to 1).

(2) There is a tendency for some strains of low virulence to increase slightly in pathogenicity in artificial culture, *e.g.* a fall of M.L.D. to $\frac{1}{5}$ th of its original figure. The amount of growth in a two-day culture varies for different strains, but not in proportion to the lethal power of the culture.

(3) The pathogenicity of young two-day "whole" broth cultures of different strains of *B. diphtheriae*, even after the strains examined have been subjected to prolonged culture on artificial media, varies in a degree which may be expressed as 1 to 50.

(4) The virulence of washed bacilli from two-day broth cultures of different strains varies at least as much as the pathogenicity of whole cultures.

(5) The amount of growth in two-day cultures of different strains varies considerably but not in proportion to the pathogenicity of the culture.

(6) The M.L.D. of toxin in a two-day broth culture varies less for different strains than does the virulence of the bacilli in the same culture.

(7) The ratio of the M.L.D. of toxin in a two-day broth culture to that of the bacilli in the same culture may frequently be expressed as $\frac{1}{4}$ or M.L.D. toxin = M.L.D. bacilli, but the M.L.D. of bacilli may be $\frac{1}{50}$ that of the toxin. The M.L.D. of bacilli was only once observed to be greater than that of the toxin.

(8) The ratio of the M.L.D. of standard bacillary emulsion to the M.L.D. of toxin from the same culture as the bacilli, varies for different strains within about the same limits as the ratio of the M.L.D. of the original emulsion to that of the toxin. The M.L.D. of standard emulsion is, however, sometimes greater than that of the toxin. $\frac{\text{M. L. D. standard emulsion}}{\text{M. L. D. toxin}}$ varies for different strains from $\frac{1}{0.25}$ to $\frac{1}{50}$.

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THE SERUM REACTIONS (COMPLEMENT FIXATION) OF THE MENINGOCOCCUS AND THE GONOCOCCUS.

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THE present communication contains a record of further attempts to establish by serological methods some criterion for the differentiation of the *Meningococcus* from the *Gonococcus*.

According to the usually accepted views of the classification of the Gram-negative cocci, an organism which occurs on what may be called neutral ground, *e.g.*, the human wrist-joint, and which resembles the *Meningococcus* in morphology and staining reactions and will not grow on artificial media except at a temperature above 22° C., must be regarded as a *Meningococcus* or a *Gonococcus* or may possibly belong to the group of Gram-negative cocci called Pseudo-meningococci, amongst which should perhaps be included the *Micrococcus pharyngitidis flavus* III. of v. Lingelsheim.

The Pseudo-meningococcus as described by Kutscher (1906), Lieberknecht (1908), Elsler and Huntoon (1909) and others, is an organism or group of organisms which occurs fairly frequently in the healthy human naso-pharynx and which resembles the *Meningococcus* culturally. According to these writers it can, however, be distinguished with varying degrees of certainty from the true *Meningococcus* by the agglutination test. My own observations (Arkwright, 1909) on the agglutination of different strains of *Meningococcus* derived from the central nervous system, lead me to place little reliance on this method as a sure test to apply to an unknown strain from a doubtful source.

Dopter (1909 II.) described strains of *Meningococcus*-like organisms occurring in the naso-pharynx, which were not agglutinated by a meningococcal serum, but which gave a complement-fixation reaction with the same serum. To these strains he gave the name of Parameningococcus. Recently the same writer (Dopter, 1911) has

described seven cases of sporadic meningitis from all of which he isolated the 'Parameningococcus.' Probably Dopter's strains of 'Parameningococcus' belong to the same category as the strains of *Meningococcus* which have been isolated by various workers (Eberle, Trautmann, Arkwright, etc.) from the meninges in cases of meningitis, but which have not been found to react with meningococcal sera prepared with other strains of *Meningococcus*.

It seems, however, reasonable to expect that it would be much easier to distinguish by serological methods the *Gonococcus* from the *Meningococcus* than the latter from an organism resembling it so closely as the *Pseudo-meningococcus*.

In spite of the usually fairly obvious cultural differences between the *Gonococcus* and the *Meningococcus* and the sharp line that is to be drawn between the sources and pathological associations of these two organisms, experience does not bear out the anticipation of wide divergence as regards serum reactions.

*Brief review of the methods advocated for the differentiation
of the Meningococcus from the Gonococcus.*

I. Cultural and biochemical tests.

(1) *Meningococci* grow on media with a much wider range of alkalinity than do *Gonococci*, and after the first few subcultures *Meningococci* will almost invariably grow well on ordinary neutral agar. This is not the case with the *Gonococcus* which requires either serum-agar or a specially prepared agar distinctly alkaline to litmus as recommended by Thalmann (1900, 1902) or a medium prepared with phosphate of soda as used by Blair Martin (1910). It can, however, easily be shown that different strains of *Gonococcus* prefer different degrees of alkalinity, and many observers have noticed that some strains of *Gonococcus* quickly become accustomed to ordinary neutral agar.

(2) On serum-agar, or agar of a suitable reaction, the colonies after 24-hours' growth in the case of the *Gonococcus* are always discrete and very small or pin point, whereas the colonies of the *Meningococcus* on the same medium are frequently confluent or, if few and discrete, are of much larger size.

These cultural characters on solid media appear to the writer to be the most constant features which differentiate cultures of the *Gonococcus* from those of the *Meningococcus*—an opinion also expressed by Wollstein (1907).

(3) Growth on carbohydrate media has been widely used and found of considerable value as a means of differentiation. Most observers, *e.g.* v. Lingelsheim, Elsler and Huntoon, Blair Martin and others have laid stress on the inability of the *Gonococcus* to ferment maltose, but I have not found this to be a constant distinguishing feature. Some strains of *Gonococcus* produce a distinct acidity when grown on serum-peptone-water or serum-agar to which pure maltose has been added (Arkwright, 1907, 1909; Wollstein, 1907; Gurd, 1908), and I have met with undoubted strains of *Meningococcus* from the cerebro-spinal fluid of cases of meningitis which did not ferment maltose. In considering these sugar reactions it must be borne in mind that the growth of *Gonococcus* on culture media neutral to litmus is usually very feeble and the production of acid from glucose is also less than in the case of the *Meningococcus*.

II. Serological tests.

I. *Agglutination.* Though many writers, v. Lingelsheim (1906), Kutscher (1906), Krumbein and Schatilloff (1908) and others have described very uniform results obtained with meningococcal serum and different strains of *Meningococcus*, this has not been by any means the universal experience of workers.

The objection to placing reliance on agglutination as a means of recognising the members of the different groups is based on two sets of facts: (1) the want of uniformity obtained with different members of the same group, *e.g.*, the *Meningococcus*, when a meningococcal serum is used (Trautmann and Fromme, 1908; Eberle, 1908; Ditthorn and Gildermeister, 1907; Lieberknecht, 1908; Elsler and Huntoon, 1909; Arkwright, 1909), and (2) the fact that some strains of *Meningococcus* will agglutinate with a gonococcal serum and *vice versâ* (Vannod, 1906; Dopter and Koch, 1908 VII.; Elsler and Huntoon, 1909; Wollstein, 1907). In the last case the experiments are further complicated by the difficulty of obtaining good uniform emulsions of the *Gonococcus*.

I found that, when working with a monovalent meningococcal serum, the number of strains agglutinated was very limited, and even when a polyvalent serum of a titre of 1-1000 made by injecting twelve different strains of *Meningococcus* was employed, strains of *Meningococcus* were easily found which were not agglutinated more highly by the specific serum than by normal serum (Arkwright, 1909).

Possibly the greater uniformity obtained by some observers has been due to the use of strains all of which occurred in the same epidemic

and to the employment of polyvalent sera made with many such strains.

It has been claimed by Dopter and Koch (1908 VII.) that by the use of the method of absorption of agglutinins a specific agglutination reaction can be demonstrated, even when a given serum agglutinates both the *Meningococcus* and the *Gonococcus* equally before absorption.

Since, however, a meningococcal serum may agglutinate some strains of *Gonococcus* but affect only a limited number of strains of *Meningococcus*, the method of absorption can only have a very restricted application.

Moreover absorption experiments (Arkwright, 1909) with different strains of *Meningococcus* and a meningococcal serum gave a differentiation between the strains of *Meningococcus* used similar to that obtained by Dopter and Koch between the *Meningococcus* and the *Gonococcus*. Torrey (1907) obtained results of the same kind in his studies on the agglutination of the *Gonococcus*.

II. By means of the Opsonic index Houston and Rankin (1907) claimed to be able to distinguish epidemic from sporadic strains of *Meningococcus*. This method according to their results would be quite useless for distinguishing the *Gonococcus* from the *Meningococcus*. Wollstein (1907) was unable to distinguish by opsonic experiments the *Gonococcus* from the *Meningococcus*.

III. The Precipitin reaction has been advocated by Dopter and Koch (1908 X.) and Dopter (1909 I.) as a means of differentiation in this group of organisms, but the experiments recorded by them were too few in number to justify the deduction of definite conclusions. Dopter carried the differentiation further by absorbing the precipitins.

IV. Complement fixation when applied for the same purpose has given very varying results. Vannod (1906) and Krumbein and Schatiloff (1908) consider this reaction specific for the *Meningococcus* and for the *Gonococcus* with their respective sera. Krumbein and Schatiloff used a polyvalent meningococcal serum and also gonococcal serum. Watabiki (1910), though not obtaining such distinct results with the two groups of cocci, maintained that the *Gonococcus* could be differentiated from other cocci by this reaction. Arkwright (1909) found this method in no way superior to agglutination as a means of distinguishing the *Meningococcus* from the *Gonococcus*. Wollstein (1907) working with monovalent sera found no distinction between the *Meningococcus* and the *Gonococcus*. Colombo (1911) has recently published a series of observations on complement fixation with these two organisms and

has found no specific difference between them. He used almost entirely polyvalent sera.

The experience of various observers as regards agglutination suggested that the use of polyvalent sera for other serum reactions in this group of organisms might be delusory. Meningococci and Gonococci appear to fall, as regards agglutination, into subgroups the members of which react among themselves, but not with the members of other subgroups of the same organism (Torrey, 1907; Arkwright, 1909). Unless, therefore, the serum used were obtained by injecting all the subgroups, it might fail to produce the reaction with the cocci which were members of the remaining subgroups and which had not been injected. A polyvalent serum might give very uniform reactions with six strains but not with a seventh or eighth. On the other hand, although a polyvalent meningococcal serum gives a reaction with some strains of *Gonococcus* as well as with some strains of *Meningococcus*, it is possible that a monovalent meningococcal serum might be obtained which had no affinities with any strain of *Gonococcus*. If such a monovalent meningococcal serum gave uniformly positive results with all strains of *Meningococcus* and negative results with all the strains of *Gonococcus* which were available, the result would be significant and the use of such a serum for the classification of new strains might be of value. Unless, however, a serum with such strictly specific activity can be obtained, serological tests are not of much value for making a final and conclusive diagnosis of a given strain of a meningococcus-like organism.

A polyvalent serum is very unlikely to fulfil these conditions and consequently a series of monovalent meningococcal and gonococcal sera were prepared with the object of testing the specificity of the complement-fixation reaction with monovalent sera.

Preparation of immune sera.

For my experiments monovalent sera were prepared by injecting rabbits intravenously with emulsions of cocci in increasing doses. Heated cocci were used first, but for the later injections living cultures were employed. Considerable difficulty was experienced in obtaining sera of sufficient strength, as the rabbits frequently died after the later doses, especially when the *Gonococci* were being used.

The strains of *Meningococcus* and *Gonococcus* used for injection were grown on horse serum agar.

Eventually three monovalent meningococcal and five monovalent gonococcal sera were obtained which gave fairly well marked complement fixation with the homologous cocci or extracts.

Experiments were also made with one polyvalent serum and one monovalent serum obtained from the horse.

Strains used.

The strains of *Meningococcus* and *Gonococcus* used in these experiments were the following:

Meningococci:

- | | |
|---------------|---|
| <i>M. XII</i> | Isolated from the meninges of a sporadic case of meningitis. |
| <i>M. 119</i> | Isolated post-mortem from the spinal cord of a case of acute epidemic meningitis. |
| <i>M. 135</i> | Isolated from the cerebro-spinal fluid of a sporadic case of meningitis. |
| <i>M. 141</i> | From the cerebro-spinal fluid of a sporadic case of meningitis. |
| <i>M. 162</i> | From the cerebro-spinal fluid of a sporadic case of meningitis. |
| <i>M. 164</i> | From the cerebro-spinal fluid of a very acute sporadic case of meningitis. |
| <i>M. 165</i> | From the cerebro-spinal fluid of an adult case of meningitis occurring in an epidemic area. |

Gonococci:

- | | |
|-------------|--|
| <i>G. 1</i> | Isolated from the vaginal discharge of a child suffering from vulvo-vaginitis. |
| <i>G. 2</i> | From a case of acute gonorrhoea in an adult male. |
| <i>G. 3</i> | From another case of acute gonorrhoea in an adult male. |
| <i>G. 4</i> | From a case of acute gonorrhoea in an adult male. |
| <i>G. 6</i> | From a case of vulvo-vaginitis in a child. |

Preparation of the antigen extract.

Extracts of the cocci were used as "antigen" for the complement-fixation reaction and various methods of extraction were tried. The following method was found to be the best of those experimented with:

An emulsion of the growth on ascitic agar in a Roux bottle was made with 10 c.c. of salt solution. This was centrifuged and the deposit made up to its original volume with salt solution. After adding a few drops of chloroform and shaking, the emulsion was left at room temperature for three or four days. The extract was then centrifuged before use. It was found that if the deposit from the last centrifuging was again made up to the original volume with salt solution and left for a further period of two to three days, a second extract as good as the first could be obtained, and by again repeating the same process, a third

and even a fourth extract could sometimes be obtained of almost undiminished value for complement-fixation experiments.

The extract was diluted eight or in some cases sixteen times before use and the specific meningococcal or gonococcal serum was diluted eight times. The largest dose of serum or extract diluted as above was 0.5 c.c. and a tube containing a double dose of serum and another with a double dose of extract were always put up as controls. Some experiments were made in which falling doses of extract were used with a constant dose of specific serum, and other experiments in which the dose of extract was constant but the doses of serum decreased in the successive tubes. The results yielded by the two methods were on the whole alike.

Haemolytic system.

The haemolytic system used consisted of sheep's corpuscles, rabbit-v.-sheep serum, and guinea-pig complement. The haemolytic serum was titrated each day with the complement and a double haemolytic dose of serum was used with 0.5 c.c. of a 1-10 dilution of fresh guinea-pig's serum.

No results are recorded unless the control tubes gave complete haemolysis.

The controls have been omitted from the tables for the sake of brevity.

The signs used in the tables indicate the amount of haemolysis which took place. Thus +++ = complete haemolysis; ++ = partial haemolysis; + = slight haemolysis; and - = no haemolysis.

Experiment I.

In Experiment I the following sera and extracts of cocci were employed :

<i>M.M.H.S. (XII)</i>	=	Monovalent meningococcal horse serum obtained by immunisation with <i>Meningococcus XII</i> .
<i>P.M.H.S.</i>	=	Polyvalent meningococcal horse serum obtained by immunisation with 20 strains of <i>Meningococcus</i> .
<i>M.E. 141</i>	=	Meningococcal extract prepared from <i>Meningococcus 141</i> .
<i>M.E. 162</i>	=	Meningococcal extract prepared from <i>Meningococcus 162</i> .
<i>G.E. 1</i>	=	Gonococcal extract prepared from <i>Gonococcus 1</i> .

The meningococcal extracts are heterologous to both sera.

The dose of extract was kept constant and used with falling doses of serum.

The results of Experiment I are shown in Table I:

TABLE I.

Sera <i>M.M.H.S.</i> (XII)	Dose	Extracts		
		<i>M. 141</i>	<i>M. 162</i>	<i>G. 1</i>
0.5	0.5	—	+	—
0.25	0.5	—	++	—
0.125	0.5	+	+++	+
0.06	0.5	++	+++	+
0.03	0.5	+++	+++	...
<i>P.M.H.S.</i>				
0.5	0.5	—	—	—
0.25	0.5	—	—	—
0.125	0.5	—	—	—
0.06	0.5	+	+	+
0.03	0.5	++	++	+

It will be seen that the monovalent meningococcal serum produced fixation of complement to an equal degree in the presence of the *Gonococcus* extract and one of the heterologous *Meningococcus* extracts, but in the presence of the other *Meningococcus* extract complement was bound only to a very slight extent. The polyvalent meningococcal serum on the other hand produces fixation of complement equally in the presence of all three extracts.

Experiment II.

In Exp. II the same two horse sera and meningococcal extracts were used, but a third meningococcal extract was also tested with these sera.

In this experiment the dose of serum was kept constant and falling doses of extract were employed.

The results obtained in Exp. II are shown in Table II. Tested in this way the difference between the reactions with *M.E. 141* and *162* were still well marked. The monovalent serum caused marked complement-fixation in the presence of only one of the extracts (*M.E. 141*) but in the presence of *M.E. 119* or *M.E. 162* it produced very slight fixation.

When the polyvalent meningococcal serum was employed, rather different results were obtained. *M.E. 119* still gave a completely negative reaction, whereas a difference was shown between *M. E. 141* and *M. E. 162*, which did not appear in Exp. I when falling doses of serum were used.

TABLE II.

Sera <i>M.M.H.S. (XII)</i>	Extracts			
	Dose	<i>M. 119</i>	<i>M. 141</i>	<i>M. 162</i>
0·5	0·5	+++	—	++
0·5	0·25	+++	—	+++
0·5	0·125	+++	—	+++
0·5	0·06	+++	+	+++
0·5	0·03	+++	++	+++
<i>P.M.H.S.</i>				
0·5	0·5	+++	—	—
0·5	0·25	+++	—	+
0·5	0·125	+++	+	+++
0·5	0·06	+++	+	+++
0·5	0·03	+++	++	+++

Experiment III.

In this experiment two monovalent rabbit sera were used and extracts of six strains of *Meningococcus* and of one strain of *Gonococcus*.

M.M.R.S. 141 = Monovalent meningococcal rabbit serum prepared with *M. 141*.

M.G.R.S. 1 = Monovalent gonococcal rabbit serum prepared with *G. 1*.

M.E. 119 = Extract of *Meningococcus 119*.

G.E. 1 = Extract of *Gonococcus 1*.

Etc.

The doses of serum were kept constant and falling doses of extract were used as in Exp. II.

TABLE III.

Sera <i>M.M.R.S. 141</i>	Extracts							
	Dose	<i>M. 119</i>	<i>M. 135</i>	<i>M. 141</i>	<i>M. 162</i>	<i>M. 164</i>	<i>M. 165</i>	<i>G. 1</i>
0·5	0·5	+	—	—	—	—	—	—
0·5	0·25	++	+	—	++	+	+	—
0·5	0·125	+++	++	+	++	++	++	+
0·5	0·06	+++	+++	++	+++	+++	+++	+++
0·5	0·03	+++	+++	+++	+++	+++	+++	+++
<i>M.G.R.S. 1</i>								
0·5	0·5	+	—	—	+	—	—	—
0·5	0·25	++	—	—	++	++	+	—
0·5	0·125	+++	++	++	+++	++	++	+
0·5	0·06	+++	+++	+++	+++	+++	+++	+++
0·5	0·03	+++	+++	+++	+++	+++	+++	+++

The results of Exp. III recorded in Table III show that the degree of complement fixation produced by the monovalent gonococcal serum and by the monovalent meningococcal serum in the presence of the same gonococcal and meningococcal extracts was almost the same. Also the meningococcal serum caused fixation of complement to the same extent whether the homologous meningococcal extract or a gonococcal extract was used. On the other hand in the presence of each of five heterologous meningococcal extracts this meningococcal serum bound complement less than when in association with the gonococcal extract.

Experiment IV.

Sera prepared with the same two cocci were used in Exp. IV as in Exp. III and in addition a gonococcal serum was employed.

<i>M.M.R.S. 141</i> =	Monovalent meningococcal rabbit serum prepared with <i>M. 141</i> .
<i>M.G.R.S. 1</i> =	Monovalent gonococcal rabbit serum prepared with <i>G. 1</i> .
<i>M.G.R.S. 2</i> =	Monovalent gonococcal rabbit serum prepared with <i>G. 2</i> .
<i>M.E. 135</i> =	Extract of <i>M. 135</i> .
<i>M.E. 141</i> =	Extract of <i>M. 141</i> .
<i>G.E. 1</i> =	Extract of <i>G. 1</i> .

The dose of extract was kept constant in this experiment and falling doses of serum were used.

TABLE IV.

Sera <i>M.M.R.S. 141</i>	Extracts			
	Dose	<i>M. 135</i>	<i>M. 141</i>	<i>G. 1</i>
0·5	0·5	—	—	—
0·25	0·5	—	—	—
0·125	0·5	+	—	+
0·06	0·5	++	+	+++
0·03	0·5	+++	+	+++
<i>M.G.R.S. 1</i>				
0·5	0·5	—	...	—
0·25	0·5	—	...	—
0·125	0·5	+	...	—
0·06	0·5	++	...	—
0·03	0·5	+++	...	+
<i>M.G.R.S. 2</i>				
0·5	0·5	—	...	—
0·25	0·5	+	...	—
0·125	0·5	++	...	—
0·06	0·5	++	...	—
0·03	0·5	+++	...	—

The results obtained in Exp. IV and shown in Table IV appear at first sight to suggest that a strain of *Gonococcus* can be distinguished from a meningococcal strain by means of a gonococcal serum used in falling doses, but that this is not a constant result is shown by Exps. V and VI. In Table IV is again shown the very uniform degree of fixation of complement obtained with a monovalent meningococcal serum, and meningococcal and gonococcal extracts.

Experiment V.

Three sera were tested in this experiment and three extracts of cocci. Falling doses of serum and constant doses of extract were used.

M.M.R.S. 162 = Monovalent meningococcal rabbit serum prepared with *M. 162*.
M.G.R.S. 3 = Monovalent gonococcal rabbit serum prepared with *G. 3*.
M.G.R.S. 4 = Monovalent gonococcal rabbit serum prepared with *G. 4*.

TABLE V.

Sera	Extracts			
	Dose	<i>M. 135</i>	<i>M. 162</i>	<i>G. 2</i>
<i>M.M.R.S. 162</i>				
0.5	0.5	+	—	
0.25	0.5	—	—	—
0.125	0.5	+	—	—
0.06	0.5	++	+	—
0.03	0.5	+++	++	—
<i>M.G.R.S. 3</i>				
0.5	0.5	++	++	—
0.25	0.5	++	+++	—
0.125	0.5	+++	+++	—
0.06	0.5	+++	+++	—
0.03	0.5	+++	+++	+
<i>M.G.R.S. 4</i>				
0.5	0.5	—	—	—
0.25	0.5	—	—	—
0.125	0.5	++	++	+
0.06	0.5	+++	++	++
0.03	0.5	+++	+++	+++

Table V shows the results of complement-fixation obtained in Exp. V. The most noticeable fact is that the gonococcal extract in the presence of either meningococcal (*162*) or gonococcal (*3*) serum produced a greater degree of complement-fixation than either of the meningococcal extracts. When, however, *Gonococcus* serum (*4*) was used, the three coccal extracts (*M. 135*, *M. 162* and *G. 2*) all produced nearly the same degree of fixation.

Experiment VI.

In Experiment VI two meningococcal sera were used, three meningococcal extracts and one gonococcal extract. Falling doses of serum and constant doses of extract were again employed.

TABLE VI.

Sera <i>M.M.R.S. 141</i>	Dose	Extracts			
		<i>M. 141</i>	<i>M. 162</i>	<i>M. 164</i>	<i>G. 6</i>
0.5	0.5	-	+	+	-
0.25	0.5	+	+	++	-
0.125	0.5	+	++	++	-
0.06	0.5	++	++	++	+
0.03	0.5	+++	+++	+++	++
<i>M.M.R.S. 162</i>					
0.5	0.5	-	-	+	-
0.25	0.5	-	-	-	-
0.125	0.5	-	-	-	-
0.06	0.5	-	-	+	-
0.03	0.5	+	-	+	-

Table VI shows the results of Experiment VI. Here again the gonococcal extract was more efficient for complement-fixation than the meningococcal extracts, even although the sera used were monovalent sera. This superiority is especially well shown in the upper part of the table where the results of fixation with a rather weak serum (*M.M.R.S. 141*) are recorded.

These experiments appear to show that though a monovalent serum produces generally a more marked complement-fixing reaction with its homologous strain of coccus than with heterologous strains, nevertheless such a serum has not necessarily any greater affinity for the other strains of the same group (*Meningococcus* or *Gonococcus*) than for strains of the other group.

It was, however, noticed (and this appears in the tables) that extracts prepared from strains of *Gonococcus* were on the whole more efficient for producing complement-fixation with a meningococcal or gonococcal serum than extracts made from strains of *Meningococcus*. Colombo (1911) arrived at a similar conclusion, both as regards the non-specificity of the complement-fixation reaction and the greater effect produced by gonococcal extract.

SUMMARY AND DISCUSSION.

The results which were obtained in the foregoing six experiments and are detailed in Tables I to VI may be summarised as follows.

Statement of complement-fixation reactions combined from all the Tables.*

Reaction of <i>M.M.H.S. (XII)</i> was	...	Positive with <i>E.M. 141</i> and <i>E.G. 1</i> . Very slight with <i>E.M. 162</i> . Negative with <i>E.M. 119</i> and <i>E.M. 162</i> .
Reaction of <i>P.M.H.S. (20 strains)</i> was		Positive with <i>E.M. 141</i> , <i>E.M. 162</i> and <i>E.G. 1</i> . Negative with <i>E.M. 119</i> .
Reaction of <i>M.M.R.S. 141</i> was	...	Positive with <i>E.M. 135</i> , <i>E.M. 141</i> , <i>E.M. 162</i> , <i>E.M. 164</i> , <i>E.M. 165</i> , <i>E.G. 1</i> and <i>E.G. 6</i> . Very slight with <i>E.M. 162</i> and <i>E.M. 164</i> .
Reaction of <i>M.M.R.S. 162</i> was	...	Positive with <i>E.M. 135</i> , <i>E.M. 162</i> , <i>E.M. 164</i> , <i>E.G. 2</i> and <i>E.G. 6</i> .
Reaction of <i>M.G.R.S. 1</i> was	...	Positive with <i>E.M. 135</i> , <i>E.M. 141</i> , <i>E.M. 164</i> , <i>E.M. 165</i> and <i>E.G. 1</i> . Negative with <i>E.M. 119</i> and <i>E.M. 162</i> .
Reaction of <i>M.G.R.S. 2</i> was	...	Positive with <i>E.M. 135</i> and <i>E.G. 1</i> .
Reaction of <i>M.G.R.S. 3</i> was	...	Positive with <i>E.G. 2</i> . Negative with <i>E.M. 135</i> and <i>E.M. 162</i> .
Reaction of <i>M.G.R.S. 4</i> was	...	Positive with <i>E.M. 135</i> , <i>E.M. 162</i> and <i>E.G. 2</i> .

* In some cases slightly different results were obtained on different occasions.

It is seen then that any attempt to classify these two groups of cocci by means of complement-binding reactions would arrange them into more or less well marked sub-groups, some of which would contain both meningococcal and gonococcal strains, and some perhaps strains from only one of these groups.

It will be noticed that extracts of three strains of *Meningococcus (119, 162 and 164)* showed an especial tendency to give negative or feeble complement-fixation reactions with heterologous meningococcal sera, and two of these strains (*119* and *162*) also gave negative reactions with some of the gonococcal sera. Though the complement-fixation reactions of the strains used in this research were not fully worked out on account of the difficulty in obtaining satisfactory sera, nevertheless the following classification appears to be indicated having regard to complement-fixation alone.

- | | | |
|-----------|------|---|
| Sub-group | I. | <i>M. (XII)</i> , <i>M. 141</i> , <i>M. 165</i> , <i>G. 1</i> , <i>G. 3</i> . |
| | II. | <i>M. 135</i> , <i>G. 2</i> . |
| | III. | <i>M. 162</i> , <i>M. 164</i> , <i>G. 4</i> , <i>G. 6</i> . |
| | IV. | <i>M. 119</i> . |

Sub-group II has affinities for sub-groups I and III, but there is little, if any, affinity shown between I and III directly. *M. 119* shows slight affinity for *M. 141*, but for no other strains.

The sub-groups are not clearly defined, but overlap and are connected with each other in various directions. For instance *G. 1*, *M. 135* and *G. 2* all appear to have common receptors, and *M. 135* and *G. 2* also have receptors in common with *G. 4* and *M. 162*, but *G. 1* shows no affinity for these two latter strains.

The explanation of these facts is not quite simple but they may be explained by assuming, (1) that several group antigens occur which are common to the Meningococcus and the Gonococcus, but only some of which are present in any given strain of coccus, and (2) that specific antigens which are peculiar to the Meningococcus on the one hand or to the Gonococcus on the other hand do occur, but are often absent in the case of any given strain.

The second assumption is perhaps unnecessary, and the first is almost equivalent to affirming the occurrence of special antigens peculiar to certain sub-groups which contain strains of both Meningococcus and Gonococcus.

The evidence, then, from complement-fixation experiments as also from other serum tests as far as they are of any value, appears to point to a closer relationship between some strains of Gonococcus and some strains of Meningococcus than between different sub-groups of Meningococcus. In fact rather the unity of these two groups than any essential difference between them, is suggested by these facts.

These considerations lend further support to the view that the most constant bacteriological characters available for differentiating the Meningococcus from the Gonococcus are the cultural characters seen when the organisms are grown on agar of different degrees of alkalinity.

CONCLUSIONS.

(1) Meningococcal sera produce complement-fixation as readily with some gonococcal extracts as with extracts of some strains of Meningococcus; whereas no reaction is obtained with some heterologous meningococcal extracts.

(2) A monovalent serum usually reacts better with an extract of its homologous coccus than with extracts of other strains of Meningococcus or Gonococcus, but a gonococcal extract sometimes gives a

better reaction with a meningococcal serum than the homologous extract does.

(3) Gonococcal sera and extracts are on the whole more potent than those prepared from Meningococci as regards complement-fixation.

(4) No satisfactory distinction between Meningococci and Gonococci can be demonstrated by means of complement-fixation tests.

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THE ACTION OF CERTAIN BACTERIA ON PROTEINS.

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THE digestion of solidified blood-serum by certain sporing bacilli is well known, and Bienstock (1899) and Rettger have shown that *B. putrificus* and other obligate anaerobic sporing bacteria destroy protein and cause putrefactive changes. Hardly any observations have been made, however, to determine whether aerobic, non-sporing bacilli and cocci have any power to break down proteid, although it is often assumed they can do so. Rettger (1906) found that bacilli of the colon group did not set up putrefactive changes in a mixture of egg and meat protein; he made no quantitative experiments as to whether any destruction of protein took place, and Dr C. J. Martin informed me that he has for long noticed that preparations of pure proteins, when left exposed to air, did not appear to be broken down by bacteria, although moulds were able to grow in them.

In the present enquiry an attempt has been made to ascertain whether certain aerobic organisms can break down either pure proteins, or solutions of protein in the presence of various nitrogenous extractives.

METHODS.

I. *Preparations of pure proteins.*

Three proteins were selected for examination, namely

- (1) Egg-albumin;
- (2) The total coagulable protein (globulin and albumin) of horse serum: and
- (3) Alkali-albumin, prepared from egg-albumin.

(1) *Egg-albumin.* Crystalline egg-albumin was prepared by Hopkins' method and dissolved in distilled water. The protein was

then precipitated with anhydrous sodium sulphate at 37° C., washed with a solution of sodium sulphate of the same sp. gr. as the filtrate, dried between sheets of blotting-paper, and redissolved in distilled water. This process was repeated many (8–10) times, until the solution was almost free from ammonia.

For the final solution, ammonia-free distilled water was used.

Most of the sodium sulphate was removed by allowing it to crystallise out at 0° C. and by subsequent dialysis of the protein solution. The solution was finally sterilised by filtration through a Berkefeld filter, previously washed with distilled water. One obtained in this way a fairly strong (2–5 %) solution of egg-albumin which served as a stock.

(2) *Serum protein.* The total coagulable protein present in normal horse serum was precipitated by the addition of anhydrous sodium sulphate, the serum being warmed to 37° C. The precipitate was washed with a solution of sodium sulphate of the same specific gravity as the filtrate, dried between sheets of blotting-paper, and redissolved in a small quantity of distilled water. This process was repeated 10–12 times until a protein solution was obtained, which was almost free from extractive nitrogen. For the final solution, ammonia-free distilled water was used; most of the sodium sulphate was removed by allowing it to crystallise out at 0° C. and by subsequent dialysis of the protein solution for an hour.

The solution, which was yellowish in colour, was finally sterilised by filtration through a Berkefeld filter and was kept as a stock.

Two or three preparations of egg-albumin and serum-protein were made in the course of the experiments, and varied somewhat as to their relative purity.

(3) *Alkali-albumin.* The material used had been prepared from pure egg-albumin by Dr C. J. Martin. It was dissolved in a 0.5 % sodium carbonate solution; normal hydrochloric acid was then added until a point was reached at which the protein began to be precipitated. The amount of free ammonia present in these solutions was estimated and used as an indication of their purity, since it is very difficult to remove all the free ammonia from such solutions by the precipitation method. The following figures were obtained for two samples:

(1) 1 c.c. of a stock solution of serum protein contained 0.000008 gm. NH_3 .

(2) 1 c.c. of a solution of alkali-albumin contained 0.000008 gm. NH_3 .

II. *Preparation of media.*

For the purpose of estimating the multiplication of bacteria in solutions of pure protein, a weak solution (0.1–0.5 %) of protein was made by diluting a portion of the stock solution of protein with ammonia-free distilled water. Various salts were added and the final composition of the medium was as follows :

Protein 0.1–0.5 %.

Sodium chloride 0.5 %.

Sodium sulphate (approximately) 0.1–0.25 %.

Calcium chloride trace	}	approximately 0.1 %.
Potassium phosphate trace		

Sulphuric acid ($\frac{N}{100}$) was added until the medium, which at first was distinctly alkaline, became very faintly alkaline to extremely sensitive red litmus paper. It was then sterilised by filtration through a Berkefeld filter, and put in test tubes, so that each contained roughly 5 c.c. of fluid.

For the chemical estimation of protein the media were prepared in the same way, except that stronger solutions of protein were used. Usually 20–25 c.c. of the medium were accurately measured with a sterile pipette into a number of small flasks, which after incubation at 37° C. for two days were tested as regards their sterility. Some were kept as controls, the remainder being inoculated with various organisms from fresh agar cultures. All the flasks were incubated at 37° C. for 7–28 days, the same time being allowed in each experiment. The amount of coagulable protein present in each flask was then estimated. The solution was poured into a small beaker, made distinctly acid to sensitive neutral litmus paper by the addition of $\frac{N}{100}$ butyric acid, and boiled for several minutes until the protein was coagulated and the supernatant fluid was perfectly clear. The coagulum was collected on a weighed filter paper, dried and weighed. In one or two experiments, the residual nitrogen was estimated by Kjeldahl's method after removal of the protein by tannic acid; and occasionally the amount of free ammonia present in the solution was determined by the vacuum distillation method.

III. *Method of determining the multiplication of organisms.*

The test tubes containing the media were incubated at 37° C. for 24 hours and then tested as regards their sterility. A small quantity of the organisms under examination was taken from a fresh agar culture by means of a fine platinum needle and added to the fluid in the test tubes. After half an hour, the tube was well shaken and two drops were taken from the test tube, by means of a capillary pipette, and dropped into 5 c.c. of sterile normal saline solution which was well shaken. This was called the *first dilution*. With the same pipette two drops were taken from the dilution and dropped into a second test tube also containing 5 c.c. of sterile saline solution, known as the *second dilution*. In some cases a third dilution was made in the same way. Five drops and one drop from the original medium and from each dilution were dropped into petri plates and ordinary agar plates were made, taking care to ensure a good mixture. After incubation of the plates for 24 hours, the colonies on each plate were counted. All the plating was done in duplicate. The test tubes containing the inoculated media were incubated at 37° C. and the number of organisms present was determined from day to day by the above method.

The same pipette was used throughout the whole of the experiment, and was dried each time before and after being used as a "dropper" so that the size of the drops should be, as far as possible, uniform. As a rule five organisms were examined in each experiment, and aseptic precautions were always taken.

Other details as to the methods used are given subsequently.

The micro-organisms used in the experiments were as follows :

- (1) *B. coli communis*, stock strain, Lister Institute.
- (2) *B. enteritidis* Gaertner, original strain.
- (3) *B. typhosus* Guy, stock strain, Lister Institute.
- (4) *B. proteus*, stock strain, Lister Institute.
- (5) *B. pyocyaneus*, stock strain, Lister Institute.
- (6) *Staphylococcus p. aureus*, from an acute abscess.
- (7) *Gonococcus*, from case of acute gonorrhoea.

The same strains were used throughout the whole investigation.

RESULTS.

I. MULTIPLICATION OF BACTERIA IN MEDIA CONTAINING ONLY
PURE PROTEIN AND SALTS.

Visible growth rarely occurred, though a faint opalescence was occasionally observed in the case of the less pure preparations of protein.

The multiplication of the organisms (as determined by plating) was influenced by two factors, namely, the purity of the medium and the number of the organisms originally added to the medium, and the actual figures obtained in the various experiments show only a general correspondence with one another.

Even the purest preparations of the proteins contained traces of ammonia, and probably other nitrogenous extractives; and some of the organisms used, notably *B. enteritidis* Gaertner, can exist and even multiply to some extent in solutions containing the merest traces of organic material. In this enquiry it was found that, when the bacterial seeding was small, some multiplication took place in all the protein media, but when a larger bacterial seeding was made, the numbers of organisms diminished in the purest protein media, whereas some multiplication occurred in the less pure protein media.

The general results are given in Tables I-III. They show that no extensive multiplication of bacteria occurred, although the seeding was usually large, and that the organisms examined grew very little better in solutions of protein than in solutions of glucose (and salts), in ammonia-free distilled water. The power to multiply was least in the case of *B. typhosus* and *Staphylococcus aureus*. In fact cultures of these two organisms on protein media were sometimes sterile, when sub-cultured after incubation for a week or more.

In a number of experiments, 0.5 to 1% glucose was added to the protein media. It seemed possible that the organisms might be able to break down protein, when a readily utilisable source of energy and of carbon was present. The addition of sugar had very little influence, however, on the growth of the organisms examined, as long as the protein medium was pure. A very faintly acid reaction of the medium to sensitive red litmus paper was sometimes observed, but in other cases no difference could be detected between the inoculated and the control tubes.

Since glucose is readily attacked by all the organisms employed, when available nitrogenous food is present, the minimal destruction of glucose (as judged by the formation of acid) in these experiments seems to show not only that the media contained very little non-proteid nitrogen, but also that very little (if any) proteid was broken down.

The fact that when comparative tests were made with the same sample of a protein medium, the number of organisms tended to become constant after a few days with either a large or small seeding (Table IV), also suggests that the organisms were living on the traces of non-protein nitrogen in the medium rather than on the protein itself. In the latter case one would expect a progressive increase in the number of organisms as more and more protein was used up, whereas, in point of fact, the number of organisms tended to decrease after three or four days. When a few drops of broth or of impure solution of albumose were added to the protein medium, all the organisms showed multiplication, and *B. coli* and *B. enteritidis* grew freely, indicating that the media contained nothing which inhibited or interfered with the growth of organisms.

Media containing gelatin.

A few observations were made with a medium containing 10% gelatin, salts and distilled water; the gelatin was not purified at all. The medium was made neutral to sensitive red litmus paper. *B. proteus*, *B. pyocyaneus* and *Staphylococcus aureus* grew very feebly on this medium; and although some liquefaction eventually took place, it was greatly delayed. When 1% glucose was added to the medium, *Staphylococcus aureus* showed very little growth, *B. proteus* and *B. pyocyaneus* grew rather more vigorously, but did not visibly liquefy the gelatin for ten days, although they liquefied control tubes of ordinary broth gelatin very rapidly.

It is probable that a medium consisting only of gelatin, salts and water, is very slowly digested by these organisms, chiefly because they grow feebly and therefore produce very little digestive enzyme. But this may not be the only factor, since the conditions which influence the activity of the extracellular proteolytic enzymes of *B. proteus* and *B. pyocyaneus* are not fully worked out; and it is not known how far an acid or alkaline reaction of the medium modifies their proteolytic power.

II. THE ACTION OF BACTERIA UPON PROTEIN, AS DETERMINED BY CHEMICAL METHODS.

The amount of coagulable protein present in protein media before and after inoculation with various organisms was estimated by the coagulation method. One or two observations were also made to determine whether the presence of bacteria in solutions of pure bacteria and salts influenced the amount of uncoagulable nitrogenous material in the solutions.

(a) *Pure proteins.*

When the solutions contained only pure protein and salts, none of the organisms examined were able to break down the protein. The results, given in Table V, show that the amounts of coagulable protein in the control and inoculated flasks were the same, allowing for the experimental error of the method. Even *B. proteus* and *B. pyocyaneus* did not destroy protein when it was pure.

The observations as to the amount of non-coagulable nitrogenous material present in control and in inoculated flasks containing solutions of pure protein showed no evidence that any destruction of protein occurred in the inoculated flasks.

(b) *Solutions of protein and nitrogenous extractives.*

A number of experiments were made to determine the effect of adding to solutions of pure protein, either albumoses, peptone, broth or other nitrogenous material. It seemed possible that, when sufficient material was present to ensure vigorous bacterial growth, the protein might be broken down. Chemical methods alone were available for this investigation.

The substances added to the protein solutions in different experiments were the following:—

(1) Ordinary peptone broth in sufficient amount to form a 0·3% solution (medium A).

(2) An impure preparation of albumose, prepared from Witte's peptone, but still containing some extractive nitrogen (medium B).

(3) Horse serum diluted and freed from coagulable protein by boiling in acid solution. The filtrate contained no coagulable protein, but gave a very faint Xantho-proteic reaction (medium C).

(4) Horse serum freed from protein by filtration through a silica filter (Martin, C. J., 1896).

(5) *Medium E.* Diluted horse serum, heated to 55° C. for half an hour to destroy complement.

Suitable amounts of sodium chloride and other salts were added to all the media, which were then *neutralised* and filtered through a Berkefeld filter. 20 c.c. were accurately measured into a number of sterile flasks; some of these were kept as controls, others were inoculated with various organisms. After incubation for six or more days at 37° C. the total coagulable protein present was estimated.

The results are given in Tables VI and VII. They show that, in the presence of an adequate supply of nitrogenous food, *B. proteus* can break down protein, but that the other organisms examined do not disintegrate it—at any rate, to any appreciable extent. No constant diminution in the amount of coagulable protein occurred, and the small differences observed in individual experiments are due to the technical difficulties of the method.

Good growth occurred in all cases, and the reaction of the solutions became alkaline as compared with the controls. The degree of alkalinity was measured by noting the number of drops of $\frac{N}{10}$ butyric acid required to render the solution neutral to very sensitive red litmus paper. The degree of alkalinity of the media (after incubation) varied with the different organisms used, being greatest for *B. proteus* and least for *Staphylococcus pyogenes aureus*.

Serum-proteins are fairly sensitive to acids and alkalies, and are gradually disintegrated in a strongly alkaline solution. But the amount of alkali formed by the bacteria used in these experiments was apparently too small to cause any appreciable destruction of the protein present in the media, even when the initial reaction of the medium was slightly alkaline to sensitive litmus paper. When a mixture of serum protein and peptone broth was used, none of the coagulable protein was destroyed, whether the initial reaction of the medium was neutral or slightly alkaline.

It may be noted also that proteins themselves can act either as weak acids or as weak alkalies, and that when present in large amount (as in blood serum) must exert a considerable influence in keeping the reaction of the medium near the neutral point.

This action, on the part of proteins, is probably of importance in modifying the way in which bacteria break down non-protein nitrogenous

substances, since it is well known that the activities of many organisms are closely dependent upon the reaction of the medium in which they are placed. The *Gonococcus*, for example, will grow well on ordinary agar, to which pure serum-protein or egg-albumin is added, although there is no evidence from chemical analysis to show that it can break down serum-protein. It will also grow on agar, to which is added the serum (deprived of all protein), but containing phosphates. Indeed, as Blair Martin (1910) has shown, strains of *Gonococcus* which fail to grow on ordinary agar will grow readily when phosphates are added.

Apparently, the *Gonococcus* requires for its existence and multiplication a medium of a particular degree of alkalinity, and the presence of proteins or phosphate serves to keep the reaction of the medium at a suitable point.

III. THE FORMATION OF INDOL FROM PURE PROTEIN.

So far as is known, indol is formed by bacteria only from tryptophane or from proteins (or polypeptides) which contain a tryptophane radicle. Whether any bacteria, when growing in media free from a tryptophane radicle, can produce small traces of indol as a product of their own metabolism is not known. In the present enquiry, *B. coli* was cultivated on some media, which responded to none of the tests for a tryptophane group; no indol could be detected in these media.

It seemed, therefore, that the formation of indol in media containing only pure protein would indicate that the indol had been formed by bacteria from the protein. The media examined were:

1. Solutions of pure egg-albumin and serum-protein.
2. These solutions with the addition of 1% urea.

The media were neutral to litmus paper and were sterilised by filtration through a Berkefeld filter. A series of test tubes containing these media were inoculated with *B. proteus* or *B. coli communis*; after incubation for 5–10 days they were tested for indol by the paradiamethylamidobenzaldehyde method. A negative reaction was obtained in all cases.

It is clear from these experiments that the organisms examined do not break down appreciable amounts of pure protein, and that *B. coli*, *B. pyocyaneus*, *B. enteritidis* and *Staphylococcus aureus* do not cause any gross destruction of protein, even in the presence of an adequate supply of non-protein nitrogenous food. There is, in fact, no evidence that these organisms can break down protein at all; possibly the protein

molecule is too large to be taken up by the organisms. It is important to remember, however, that exceedingly small quantities of nitrogenous food are needed to maintain bacterial life and permit of some multiplication; and it is conceivable that the organisms examined may be able to break down very minute traces of protein, traces so minute as to escape detection by the methods employed.

CONCLUSIONS.

(1) When solutions of pure egg-albumin, serum-protein or alkali-albumin are inoculated with the organisms studied in this enquiry, the bacteria diminish in number when the seeding is large, but multiply to some extent when the seeding is small. The evidence goes to show that the multiplication observed with small seedings is due to the presence in the medium of traces of non-protein nitrogenous substances, and that the bacteria do not use protein as food.

(2) There is no chemical evidence that the organisms which have been studied are able to break down pure egg-albumin and serum-protein; but such very minute amounts of nitrogenous food suffice to maintain bacterial life that, with the methods available, one cannot be certain that the organisms did not disintegrate and use as food very small traces of protein.

(3) The organisms examined (with the exception of *B. proteus*) do not break down appreciable quantities of egg-albumin and serum-protein, even in the presence of sufficient non-protein nitrogenous food, to ensure vigorous bacterial growth.

I wish to acknowledge my indebtedness to Dr C. J. Martin for constant help and advice in the course of this enquiry.

TABLE I.

The organism examined was *B. typhosus*.

The figures represent the number of organisms actually counted on the plates made in duplicate from the various dilutions of the medium.

(a) *Egg-albumin*.

Dilution examined	Period of incubation in days			
	0	1	2	6
Medium itself, 5 drops	Uncountable	—	—	—
„ 1 drop	„	—	Uncountable	—
1st dilution, 5 drops	243, 239	222, 190	626, 528	298, 310
„ 1 drop	60, 50	46, 38	201, 158	80, 75
2nd dilution, 5 drops	—	—	—	5, 1
„ 1 drop	—	—	—	1, 0

(b) *Serum-protein*.

Dilution examined	Period of incubation in days		
	0	1	2
1st dilution, 1 drop	Too many to count	405, 492	192, 212
2nd dilution, 5 drops	609, 490	16, 18	10, 10
„ 1 drop	55, 90	5, 3	1, 3
3rd dilution, 5 drops	7, 21	1, 0	1, 0
„ 1 drop	1, 1	0	0

TABLE II.

The figures represent the number of organisms present in one drop of the medium, which was examined at the time of inoculation and on subsequent days.

In the case of most of the organisms, two experiments are recorded, one with a small seeding and one with a large seeding.

(a) Egg-albumin.		Period of incubation in days				
Organism examined		0	1	2	3	4
<i>B. typhosus</i> (1)	...	6,025	5,150	15,150	—	—
" (2)	...	56,125	—	20,375	—	14,375
<i>B. coli communis</i> (1)	...	11,125	—	—	35,500	—
" (2)	...	495,875	—	153,750	—	30,000
<i>B. enteritidis</i> (1)	...	185,625	—	245,625	—	232,500
" (2)	...	37,500	—	144,375	—	—
						6 days 7,500.
						6 days 25,250.
						8 days 26,250.
(b) Serum-protein.		Period of incubation in days				
Organism examined		0	1	2	3	4
<i>B. typhosus</i> (1)	...	1,029,375	31,875	18,750	—	—
" (2)	...	34,250	45,375	—	17,125	—
<i>B. coli communis</i> (1)	...	335,575	123,750	50,625	—	—
" (2)	...	200,000	60,125	57,500	20,250	—
<i>B. enteritidis</i>	...	468,750	288,750	264,375	—	211,875
<i>B. proteus</i> ...	...	840,000	520,000	393,750	—	—
<i>Staphylococcus aureus</i> (1)	...	2,148,750	1,312,500	161,250	—	—
" (2)	...	228,750	157,500	86,250	—	—
						No indol, 7 dys.
(c) Alkali-albumin.		Period of incubation in days				
Organism examined		0	1	3	6	
<i>B. typhosus</i> ...	...	150,000	59,250	32,875	28,875	
<i>B. coli communis</i> ...	...	143,750	—	153,750	112,500	
<i>B. enteritidis</i> ...	...	843,750	—	453,750	61,875	
<i>B. proteus</i> ...	...	71,000	—	150,000	60,000	
<i>Staphylococcus aureus</i> (1)	...	90,000	59,415	56,250	—	
" (2)	...	52,250	—	29,375	2,000	
(d) Serum-protein + 1 % glucose.		Period of incubation in days				
Organism examined		0	2	4	15	
<i>B. coli communis</i> ...	...	843,750	423,125	153,750	—	15 days. No indol.
<i>B. enteritidis</i> ...	...	1,350,000	1,025,625	468,750	900 (approx.)	
<i>B. proteus</i> ...	...	1,428,750	480,000	69,375	—	15 days. No indol.
<i>Staphylococcus aureus</i> ...	...	300,000	205,500	44,500	—	
(e) Alkali-albumin + 1 % glucose.		Period of incubation in days				
Organism examined		0	2			
<i>B. coli communis</i> ...	...	77,000	98,500			5 days, slightly acid to litmus.
<i>B. typhosus</i> ...	...	613,125	570,000			5 days, neutral to litmus.
<i>Staphylococcus aureus</i> ...	...	1,076,250	973,125			

TABLE III.

The organism used was *B. coli communis*.

The figures represent the number of organisms present in one drop of the medium at the time of inoculation and on subsequent days.

Medium used	Period of incubation in days				
	0	1	2	3	4
(1) Normal saline solution	12,300	—	—	425	—
(2) 1 % glucose + salts ...	33,875	221,250	—	119,250	—
(3) Egg-albumin (a) ...	11,125	—	—	35,750	— 6 days, 25,000.
(4) „ (b) ...	496,875	—	—	153,750	— 6 days, 30,000.
(5) Serum-protein (a) ...	330,000	123,750	48,700	—	—
(6) „ (b) ...	71,375	55,125	—	30,625	— 8 days. No indol.
(7) Serum-prot. + glucose	843,750	—	423,125	—	153,750
(8) Alkali-albumin ...	143,750	—	153,750	—	— 11 days, 112,500.
(9) Alk.-alb. + glucose ...	20,000	—	250,000	—	—

TABLE IV.

The medium used was pure serum-protein. The organism examined was *B. coli communis*. The figures represent the number of organisms actually counted on the plates.

I. Small seeding.

Dilution counted	Period of incubation in days		
	0	1	3
1st dilution, 1 drop	13	1212	810 9 days. No indol.
2nd dilution, 5 drops	1	69	61
„ 1 drop	1	10	12

II. Large seeding.

1st dilution, 1 drop	1168	1000 (approx.)	1015 9 days. No indol.
2nd dilution, 5 drops	66	97	103
„ 1 drop	10	25	28

TABLE V.

The action of bacteria upon solutions of pure protein and salts.

In all cases the initial reaction of the medium was as nearly as possible neutral. Control experiments were always made.

The figures represent the weight of coagulable protein in grammes present in the various flasks. Each flask contained 20 c.c. of the protein solution.

The period of incubation varied from 8–24 days.

Organism examined	Serum-protein		Egg-albumin	
	1+glucose	2	1	2
Control (1) ...	0·0942*	0·1097	0·1488	0·0944
„ (2) ...	0·0993	0·1115	0·1500	0·0944
<i>B. coli communis</i> ...	0·1008	0·1084	0·1470	—
<i>B. enteritidis</i> Gaertner	0·0963	0·1084	0·1455	—
<i>B. proteus</i> ...	0·1010	—	0·1480	—
<i>B. pyocyaneus</i> ...	0·0992	0·1083	0·1492	0·0964
<i>Staphylococcus aureus</i>	0·1013	0·1076*	0·1507	—
<i>Gonococcus</i> ...	0·0972	—	—	—
Period of Incubation	12 days	24 days	14 days	8 days

* Slight loss of protein.

TABLE VI.

The action of bacteria on solutions containing serum-protein, salts, and various non-coagulable nitrogenous substances.

In all cases the initial reaction of the medium was as nearly as possible neutral. Control experiments were always made.

The figures indicate the weight of coagulable protein present in the various flasks. The period of incubation varied from 9–28 days.

Each flask contained 20 c.c. of the solution.

Organism examined	A Alkaline	A	C	C	E	
		Protein + peptone-broth neutral	Serum-prot.	+ filtr. + salts	Horse-serum	
			1	2	1	2
Control (1) ...	0·0886	0·1470*	0·0780	0·1109	0·1884	0·3563
„ (2) ...	0·0860	0·1519	0·0776	Lost	0·1806	0·3507
<i>B. coli communis</i> ...	—	0·1554	0·0778	—	0·1710	0·3485
<i>B. enteritidis</i> Gaertner	0·0948	0·1569	0·0792	0·1120	0·1758	0·3469*
<i>B. pyocyaneus</i> ...	0·0906	0·1565	0·0542	0·1130	0·1640	0·3558
<i>B. proteus</i> ...	0·0527	—	0·0295	—	0·1322	0·2986
<i>Staphylococcus aureus</i>	—	—	0·0738	—	0·1906	—
<i>Gonococcus</i> ...	—	—	—	0·1102	—	—
Period of Incubation	—	9 days	10 days	17 days	9 days	28 days

* Slight loss of protein.

TABLE VII.

The action of bacteria on solutions containing egg-albumin, salts and various non-coagulable nitrogenous substances.

In all cases the initial reaction of the medium was as nearly as possible neutral. Control experiments were always made.

The figures indicate the weight of coagulable protein in grammes present in the various flasks. The period of incubation varied from 6-10 days.

Each flask contained 20 c.c. of the solution.

Organism examined	A Egg-albumin+ peptone-broth	B Egg-albumin+ albumose	C Egg-albumin+ filtered-serum
Control (1)	0·1923	0·1209	0·1392
„ (2)	0·1902	0·1162	0·1406
<i>B. coli communis</i> ...	0·1968	0·1210	0·1419
<i>B. enteritidis</i> Gaertner	0·1918	0·1199	0·1396
<i>B. pyocyaneus</i> ...	0·1861	0·1200	0·1592
<i>B. proteus</i>	—	—	0·1042
<i>Staphylococcus aureus</i> ...	0·1938	0·1188	—
Period of Incubation	6 days	6 days	10 days

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AN OUTBREAK OF DYSENTERY.

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*(Medical Officer of Health for Paddington.)*I. *History of the outbreak.*

ON February 10th, 1911, the attention of the Medical Officer of Health was called by the practitioner in charge to an outbreak of diarrhoea in a family named R. residing in Paddington. The note made at the interview was to the effect that cases had occurred on the 8th, 9th, and 10th (2 cases) of the month, the four patients having violent diarrhoea and passing much blood. The onset was quite sudden in every case. It was thought that "food poisoning" could be excluded, and the main object in directing attention to the outbreak was to ascertain if any defective drainage existed at the house. Incidentally it may be remarked that the house drainage, etc. had been completely overhauled in 1907 and that no defects existed in February last except one leaky joint on a waste pipe.

Inquiries were at once set on foot and it was found that the cases of diarrhoea were more numerous than stated.

The house is an old one, originally a good class dwelling, one of a group of houses known to one of us since the '60's when they were occupied by well-to-do families. The house at the time of the inquiries was let out in six tenements, the occupants numbering 30.

Cases of diarrhoea were found to have occurred in the R. and H. families who were much together (Mr R. keeps a coffee-stall and Mr H. assists him). No history of any attacks could be obtained among the other occupants of the house.

Half basement (2 rooms) and } 2nd floor (attic) front (1 } room);	{ R. family; man, wife and 10 children, ages 16 yrs.—3 mths., twins.
Ground floor (2 rooms) and } 2nd floor (1 room);	{ L. family, 3 adults and 4 chil- dren.
First floor back (1 room);	{ H. family, 2 adults and 3 chil- dren.
First floor front (1 room), and } 2nd floor (1 room);	X. family, 3 adults.
First floor (1 room);	One adult (away).
Second floor front (1 room);	Two adults (away).

A further case was found in a family named J. residing in the neighbourhood, the patient being a cousin of the R. children and much with them.

Unfortunately, as so frequently occurs, rather more than a fortnight had elapsed between the date of the first attack and the commencement of the inquiry (February 11th). Numerous visits of inquiry elicited the following history.

(1) R. R., f. aet. 5, taken ill January 26th with sickness, severe abdominal pain, much diarrhoea with blood. She was seen by a Dr C. on the next day and was well again on the 29th.

(2) E. R., f. aet. 5, taken ill with same symptoms on the 29th. She was seen by Dr C. and appears to have recovered by February 5th.

(3) C. R., f. aet. 7, was attacked about 3rd February. She had no sickness and was still ill on the 11th.

(4) W. J., f. aet. 3, attack of same kind commencing on February 4th; attended St Mary's Hospital as out-patient.

(5) A. R., m. aet. 2. Attack commenced February 5th. His attack, one of the severest of the series, was attended with collapse. He was still under treatment on the 11th.

(6) B. H., f. aet. 1, bad diarrhoea on February 5th (no medical attention).

(7) E. R., m. aet. 3 months,—one of twins—was taken ill on the 8th or 9th.

In addition to the seven well-defined attacks there were two others in which the history was less definite.

I. R., f. 11, slight diarrhoea ($\frac{1}{2}$ day) on February 5th.

T. R., m. 14, "ailing" from about February 10th. No history of diarrhoea could be obtained.

Of the R. children, three escaped attacks, viz.

J. R. (m. 16), J. R. (m. 10) and the other twin.

None of the parents in the R., H. or J. families were ill.

The fact that the cases occurred very nearly singly and in succession may be taken as conclusive evidence that "food poisoning" was not the causal factor. Further, the fact that all the children who were ill were closely associated—almost living together—while other children living in the house but not associating with the R., H., or J. families, escaped, points to personal infection, or transfer of infection through common use of closet, etc.

II. *Bacteriological examination.*

1. *Faeces and urine.* On February 14th, 1911, samples of faeces and urine from two patients (E. and R. R.), and faeces only from a third patient (L. H.) were received. The material was plated out on lactose-neutral-red-bile-salt-agar, and the white colonies were picked off, and examined as regards their morphological and cultural reactions. The faeces and urine of one patient (E. R.) yielded a negative result, as did also the urine of R. R. The faeces of R. R. and L. H., however, yielded numerous colonies of non-motile bacilli which had the following characters:

1. Production of acid on glucose, mannite, sorbite, arabinose, galactose and laevulose.

2. No visible fermentation of lactose, cane sugar, dulcitol, adonitol or inulin, salicin, raffinose, maltose, xylose or glycerin.

3. Production of indol.

4. Milk showed initial acidity, but became alkaline in fourteen days.

On February 21st samples of faeces were received from L. H., E. R., R. R., T. R., L. A. R., and W. J.; all these children had suffered from clinical symptoms of dysentery. The faeces were soft and shiny in some cases, hard and green in others. On examination, bacilli identical with those first described were obtained from the faeces of T. R. and E. R.; the others gave a negative result.

Finally on February 25th and March 1st samples of faeces were obtained from the parents of the R. family and from Mr H. They had not been ill, and no dysentery bacilli were found in their stools.

The agglutination reactions of the organisms are shown in Table I. All the strains were tested with sera obtained from rabbits immunised

with *B. dysenteriae* (Flexner), *Bacillus* "Y" of Hiss and Russell, and *B. typhosus* respectively. It will be seen that the strains were not agglutinated by the Flexner serum in such high dilutions as *B. dysenteriae* (Flexner) or the "Y" bacillus, but they were agglutinated by the "Y" serum to the same extent as *B. dysenteriae* (Flexner) and *Bacillus* "Y." They were agglutinated in low dilutions only by the typhoid serum. These results correspond with those obtained by Morgan (1911) in the case of British strains of *B. dysenteriae*.

TABLE I.

Agglutination tests. Macroscopic method.

Organisms examined	<i>B.</i> "Y" serum	<i>B. Flexner</i> serum	<i>B. typhosus</i> serum titre 4,000
<i>B. dysenteriae</i> , Flexner	2,000	5,000	—
<i>Bacillus</i> "Y"	10,000	5,000	—
L. H.	1,000	1,000	800
E. R.	1,000	1,000	—
T. R.	1,000	1,000	—
R. R.	1,000	1,000	100

Absorption tests were not made as they seem to be of little value for the differentiation of this group of bacilli.

2. *Samples of blood.* Blood was obtained from three patients, namely R. R., T. R., and L. H., and tested as regards its agglutinating power for *B. typhosus* and *B. dysenteriae* (Flexner). The results are given in Table II.

TABLE II. *Agglutinating power of patients' serum.*

Microscopic method. Controls were used in each case.

Patient	Organism tested	
	<i>B. typhosus</i>	<i>B. dysenteriae</i> (Flexner)
R. R.	Positive reaction	Positive reaction (1-100)
T. R.	Negative "	" "
L. H.	" "	" "

In the case of R. R. the reaction was more complete with *B. dysenteriae* than with *B. typhosus*.

Sera, prepared by injecting *B. dysenteriae* into rabbits, usually contain secondary agglutinins for *B. typhosus*, so that the reaction of the serum of R. R. with both typhoid and dysentery is not surprising.

The presence of *B. dysenteriae* in the faeces of the patients, and the strong agglutination of this bacillus by the serum of three patients,

furnish evidence that this bacillus was the cause of their illness. Apart from Asylum dysentery, outbreaks of dysentery in this country appear to have been very rarely observed; the only instance previously recorded was a fatal case occurring in a child in London, in which Marshall (1909) obtained from the faeces a bacillus identical in cultural and agglutination reactions with *B. dysenteriae* (Flexner).

Morgan (1911), however, has recently examined a large number of strains of dysentery bacilli, which had been isolated by Ledingham from the faeces of typhoid convalescents and from other sources, and his observations seem to indicate not only that dysentery bacilli of the Flexner type are more often present in the faeces of healthy persons than has been hitherto supposed, but also that slight differences in the fermentation reactions of different strains are of minor importance.

On receiving information of the results of the bacteriological examination, inquiries were made to ascertain if any members of the affected families had been abroad or whether there had been any contact with persons recently arrived from foreign parts. Neither Mr nor Mrs R. had ever been out of the country, nor had any visitors from abroad. Mr H. had served in the Royal Army Medical Corps, being stationed at the Cambridge Hospital, Aldershot, where patients from all parts of the world are received. He had, however, left the Army some ten years ago. His faeces, moreover, were free from the *B. dysenteriae*.

It is worth noting that positive results were obtained twice from the first patient (E. R.), an interval of one week intervening between the taking of the specimens; also that T. R., in whose faeces the *B. dysenteriae* (Flexner) was found, and whose blood gave a positive reaction with the same bacillus, was not ill to the same severity as were other children from whose faeces the bacillus could not be isolated. T. R.'s trifling attack occurred ten days prior to the day on which his specimen was taken.

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DEMONSTRATION OF SPECIMENS RELATING TO THE CULTURE OF THE LEPROSY BACILLUS.

*In the Section of Tropical Medicine at the Annual Meeting of the
British Medical Association, Birmingham, July, 1911.*

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So many claims have already been advanced in relation to the culture of the leprosy bacillus that I certainly should not have added my observations to the list had I not felt that I was in the position to demonstrate a connecting link between the conflicting evidence of several observers. I hope also to exhibit specimens and refer to certain biological tests not as yet carried out in this connexion.

Hansen's *Bacillus leprae* is commonly considered the causative agent of leprosy in the human being, though we must admit that so far we have no definite and absolutely conclusive proof as to its primary etiological rôle. Profeta's inoculation of himself and others have been negative; Arning's infection of a criminal appears to be open to criticism; the same applies to Coffin's case; and thus we might for a moment consider whether the primary lesion is not caused by a germ so far totally unrecognized, whilst the acid-fast rods seen in such enormous quantities in leprosy affections are but harmless superadded saphrophytes.

The recent experience with swine fever (hog cholera) shows us that the constant or apparently constant presence of a germ in a definite disease does not conclusively prove its etiological rôle. However, I believe we are quite safe in taking the *Bacillus leprae* of Hansen to be the causative agent of leprosy in the human being.

It is not so very long ago that every acid-fast rod was considered to be *B. tuberculosis*, and, if not that, either the lepra or the smegma bacillus. The discovery of Lustgarten's so-called syphilis bacillus and the work of Lydia Rabinowitsch soon showed that acid-fast rods are ubiquitous, and can only be distinguished by careful and extensive cultural, experimental, and serological tests.

Without taking into consideration the many acid-fast germs found in earth, on grass, in milk and dung, in birds, reptiles and fishes, we may call to our mind that atypical acid-fast rods—that is, not tubercle or lepra—have been found in the sputum by Zahn, Pappenheim, Fraenkel, Moeller, Marzinowsky, Rabinowitsch, Lichtenstein, Lubarsch. Dr. Newham, of the London School of Tropical Medicine, has shown me specimens of acid-fast rods from the sputum of an Indian, which morphologically are certainly not tubercle bacilli.

Acid-fast rods were found in an ovarian cyst by Dietrich; in a pleural exudate by Kedrowsky; on the tonsils by Marzinowsky, Beck, Moeller; in the slime of the pharynx, in the coating of the tongue and teeth by Moeller, in sebaceous glands by Gottstein, Matterstock; in the faeces by Craemer, Ferran, Strasburger, Mironescu, Weber; in gangrenous tissues by Folli; in a phlegmon of the foot by Karlinski. This author, and also Moeller, found acid-fasts in the nasal mucus; in fact, Karlinski examined 215 patients and 20 healthy persons, and in 21 he found acid-fasts in the nose, namely, in 5 cases of syphilis, 4 cases of malaria, 2 of common cold, and then in 10 healthy subjects. He cultured the germ and infected his own nose with it. The infection lasted five days, and then passed away without leaving any further traces.

These observations have a certain practical importance. Leprosy bacilli have been cultured from the nasal mucus, the diagnosis of leprosy has often to be made from the presence of acid-fast rods from the nasal discharge, and in fact the frequency of the bacilli in the nose of leprosy patients led Sticker to formulate the hypothesis that the primary lesion was always to be found in the nose.

It must, however, not be forgotten that Uhlenhuth and Westphal found *post mortem* in a case of maculo-anaesthetic leprosy, acid-fast bacilli in enormous quantities underlying the mucous membrane of the nose, and even though it cannot be said to be proven so as not to admit the least possible doubt, still we may safely take it that they were all lepra bacilli.

Having now mentioned the pitfalls that beset the path of anybody attempting to culture Hansen's bacillus, I shall report on my results and experiences.

Some years ago, whilst I was Pathologist to the Seamen's Hospital at Greenwich, Sir Malcolm Morris kindly allowed me to make some culture attempts from a case of leprosy under his care. A nodule was excised, half of it was used for histological specimens, the other half was put into a glycerine-agar tube with a little glycerine broth at the bottom of the slope. After four weeks a diphtheroid rod grew from the nodule. I stained it according to Ziehl-Nielsen, the germs took readily the blue stain. I was much disappointed, and not having a Certificate A. for the purposes of animal experimentation, allowed the culture to die out, whilst I awaited the appearance of acid-fast colonies, which, however, were not forthcoming.

On my return from Africa my attention was again

brought to bear on the question of the culture of the germ of leprosy. Of all the communications on the subject the papers of Kedrowsky seemed to me to be the most complete and his work appeared to me to give a most satisfactory explanation of the conflicting results of various workers in the same field. I must add that though his first communication was published so long ago as 1901 it has not been much taken into consideration by subsequent workers, however similar their results may have been to his.

I went to Russia, and whilst carrying out some researches on spirillum fever worked in Professor Kedrowsky's laboratory. He very kindly showed me all his sections and gave me one of his leprosy cultures, the one you now see in the rack on the table.

On my return to England, bearing in mind that, according to the observations of Herrman and Abraham, the leprosy bacillus need not always be acid-fast, I made another attempt to culture the germ from another case of leprosy which Sir Malcolm Morris put at my disposal at the London School of Tropical Medicine.

The media I used this time were: Placental extract-agar, horse serum-nutrose-agar, to which I added 2 per cent. ground-up smegma bacilli, according to Twort's method. From an excised nodule I cultured again on several of these tubes the diphtheroid I have already mentioned, or at least one very similar to it. This I found to be acid-resisting, though not acid-fast. On staining for ten minutes with warm carbol-fuchsin, it resisted bleaching for five seconds with 1 per cent. H_2SO_4 , and, what is more important, the subsequent treatment with dilute Loeffler without turning blue. Any other germ, acid-fast, of course, excepted, is decolorized by this treatment.

I have placed specimens of this bacterium under microscopes Nos. 1 and 2. As soon as I had a sufficient amount of this culture I injected $\frac{1}{2}$ of a c.cm. of emulsion into the peritoneum of a mouse. The mouse died in three weeks' time, I believe of a diplococcal infection. No marked macroscopic lesions could be detected, but smears from the liver and spleen showed along with the diplococcus a small quantity of acid-fast rods, which could not be bleached by 10 per cent. HNO_3 , after three minutes of warm carbol-fuchsin. A small whitish lump in the peritoneum about the size of a pin's head consisted of markedly acid-fast bacteria, which had retained their diphtheroid appearance, and also of a few cells. This I consider to be simply an organized clump of germs. I have placed the corresponding slide under microscope No. 3. I collected also all the enlarged glands I could find in the abdomen, and, having imbedded them, cut sections. The result you can see under microscope No. 4. The resemblance to human leprosy glands seems to me to be most striking, but still more striking is their resemblance, amounting to identity, to the lesions found in genuine spontaneous leprosy of the rat. Compare microscopes Nos 5 and 6, no difference can be made out between the appearance of both sections. One has been produced experimentally, the other is a slide kindly lent

me by Dr. H. R. Dean, of the Lister Institute, and originates from a rat found to be suffering from rat leprosy at Ipswich.

From another nodule from the same patient I grew a non-acid-resisting streptothrix. Notwithstanding the deceptive appearances it presented under the microscope, I fear it was a simple contamination. I have, however, injected it into a rat, and am awaiting results.

In this connexion I may mention that quite lately Beauchamp Williams has cultured from the lepromata of 17 cases in India a non-acid-fast diphtheroid which turned acid-fast on being cultured in amoebae according to Musgrave and Clegg's method. His animal experiments do not appear to have given results comparable with those I am demonstrating, but anyhow he advances the view that leprosy may be caused, perhaps, by several strains of an extremely pleomorphic streptothrix, which under certain circumstances may be :

1. A non-acid-fast streptothrix with interlacing filaments.
2. A non-acid-fast diphtheroid bacillus, which is in reality a streptothrix and capable of becoming acid-fast under certain defined conditions.
3. A definite acid-fast filamentous streptothrix; and
4. An acid-fast bacillus which is the broken-down stage of the streptothrix.

It will be seen that this extremely latitudinarian point of view covers a very extensive ground. I am ready to admit that the germ of leprosy as seen in rats appears to be a connecting link between the corynebacteria and mycobacteria according to Lehmann and Neumann's definition. To suit Williams's point of view it would have to be an actinomyces also. We need further observations to settle the point. I may add that Williams got quite a marked rise of temperature by injecting into lepers vaccines made from his cultures.

Returning to the results of my attempts to culture Hansen's bacillus, I should like to mention that on a tube of horse-serum-nutrose-agar with 2 per cent. ground up smegma bacilli there grew a small brown colony; this I transferred to another tube, and then to potato. Unluckily the potato got contaminated and a fungus ruined my success. This colony consisted of acid-fast rods, absolutely identical in appearance to the bacillus seen in lepromata. The corresponding slide is under microscope No. 7. I still hope to be able to save this culture or to renew the result in course.

So far my researches are only the confirmation of the observations made by several others working on similar lines, the most extensive experiments having been first published by Kedrowsky.

A point I cannot explain so far is why the cultures I make from inoculated animals are always acid-fast, whilst the cultures taken from man only very exceptionally give the acid-fast variety, but much more frequently the

diphtheroid, simply acid-resisting form. I suppose this is due to the fact that human cases are of much longer standing, chronic forms in fact, whilst the animals experimented upon are killed at short intervals. A point in favour of this view is that in genuine, spontaneous rat leprosy, which is also a very chronic disease, a diphtheroid can easily be cultured from the lesions. This has been done by George Dean and also H. R. Dean.

The sections under the remaining microscopes show the lesions resulting from the injection of the acid-resisting diphtheroid after it has passed through a mouse. You will note the enormous deposits in the various organs attacked. The morphology of the acid-fast germs, their position in the cells, the slight, minimal reaction they cause in the surrounding tissues, the scarcity—extreme scarcity—of giant cells of the Langhans type, the absence of caseation and vascular sclerosis, seem to me to definitely prove that we are not dealing with a tubercle bacillus of any known strain. Many features of the germ bring it very near to the smegma bacillus, which also grows like a diphtheroid, is varying in its acid-fast properties, is not markedly pathogenic to animals, and under certain circumstances also grows in the same peculiar wrinkled fashion on horse-serum-nutrose-agar. The most marked difference is, however, present in the rate of growth. Smegma takes two days to grow on most media, even simple agar will do, while this acid-fast germ, after passage through rats, took two months to reach this stage, and does not grow hardly at all, except on the media I have mentioned. As an example I can show a tube of egg-medium-tubercle-bacilli, which Dr. Twort kindly put at my disposal. You will see that after three months in the incubator a growth, it is true, has taken place, but it is exceedingly thin.

In view of the similarity of the *B. leprae* to the *B. smegmae*, several experiments were carried on with the latter. Two c.cm. of a thick emulsion injected intravenously into a rabbit caused very striking alterations within three weeks. The glandular tissue of the breast got diffusely infiltrated (this also happens in males in cases of human leprosy), nodules appear on the skin in several spots, and the left hind leg was completely paralysed. The kidney was honeycombed with caseous nodules. If you will examine the sections under the last microscope you will, however, notice that no possible resemblance whatever can be made out to the lesions caused by the germs cultured from lepers under the preceding instruments.

In the rack on the table opposite you I am demonstrating a series of cultures of various acid-fast organisms, which I have put up for the sake of comparison. You will agree that the difference in appearance is marked; the rate of growth is still more so. Passing now to the various serological tests employed to determine the relationship to each other, and the degree of immunity conferred by these various germs.

Again the first experiments were made with the smegma bacillus, because it is a definite well-known bacterium, is easily grown, and can easily be procured; its properties and life-history are fairly well known and have been fairly well studied. I found that by intra-abdominal injections of killed cultures into rabbits a serum could be produced which agglutinated the smegma bacillus in the dilution of 1 in 20 to 1 in 30.

I then passed a bacillary emulsion through a Berkefeld filter, got a clear filtrate, mixed it in varying proportions of the anti-serum, and after twenty-four hours in the incubator found a precipitate at the bottom of the tubes in all dilutions up to 1 in 64. I then tried the complement-deviation test; 1 in 1 and 1 in 2 were negative, but all other dilutions down to 1 in 32 were positive—that is, no haemolysis took place. Once I had these results I proceeded to immunize a rabbit against the germ cultured from the case of human leprosy. After three injections at five days' interval, the serum being taken the twenty-fifth day, I got an antibody capable of agglutinating its antigen 1 in 20 to 1 in 30, precipitating 1 in 30 to 1 in 40, and giving the complement-deviation test within these limits.

I also had several negative results, and in two cases steaming for one hour at 60° C. was not sufficient to kill the cultures. All the same, the reaction came off to a slighter degree (1 in 8) in the infected rabbits.

In one case I tested antismegma bacillus serum against the germ cultured from the case of leprosy in a precipitation test. Slight cloudiness appeared in all dilutions down to 1 in 32, but no definite precipitation took place.

I am still repeating these experiments, and intend to obtain serums with higher values, and, if possible, absolutely specific for their relative antigen.

I also produced a bacterial extract by passing the condensation water of a nutrose-agar-horse-serum slope which contained a great number of bacilli through a Doulton filter. The extract was rubbed into the skin of a case of leprosy under the care of Sir Malcolm Morris. The same and following day the man's temperature rose to 100° and 99° F. No local reaction appeared. The patient had already undergone a course of Nastin treatment. An extract made in the identical fashion of Koch's Alt-tuberculin has also been prepared, and I intend carrying out therapeutic experiments with it.

All these serological tests have so far been carried out in a tentative fashion, as the technique must always be adapted to each individual case. The results obtained so far, I believe, can be said to be full of promise.

The paramount difficulty in connexion with the culture of germs from cases of leprosy lies not in the culture itself, but in clearly proving the relationship of the bacteria cultured to the disease they are alleged to cause. If, as I believe to have shown, these acid-fast germs can be made to give definite reactions, it is only a question of time and patience to perfect the technique to the point of getting an individual specific result.

Now a few words regarding the connexion of human and rat leprosy. This rat disease was first studied by

Stephansky at Odessa and George Dean in London, later by Lydia Rabinowitsch in Berlin, Tidswell in New South Wales, Jitoyo and Sakaki at Kobe (Japan), Wherry in the United States. It has again been found in rats at Ipswich by Petrie and Macalister.

It is quite possible that the human and rat disease are to each other in the same relation as bovine and human tuberculosis, but, on the other hand, we cannot exclude that they are caused by the identical germ. Have we not a striking analogue in plague? Cannot plague smoulder among rats for years and years and then suddenly appear as an epidemic? May not this chronic disease in rats be the survival of the endemic leprosy of the Middle Ages? George Dean, in his paper on rat leprosy, seems to take this point of view into consideration, but concludes that, so far, we have not sufficient evidence to decide the question. I believe I can bring some further proof in favour of the identity of human and rat leprosy. If you will examine the tissues under the microscopes you will be unable to distinguish the genuine, spontaneous leprosy of the rat from the experimental variety in the mouse and rat which was produced by a germ cultured from man.

I was very much struck on reading G. Dean's paper, and finding that the diphtheroid he cultured from the leprosy rats was agglutinated by rat and also by human leprosy serum in the dilution of 1 in 20 to 1 in 30. Should all these peculiar similarities be regarded as fortuitous?

Regarding the transmission of leprosy, I am trying to collect evidence to show that, according to the manner in which the infection takes place, the disease is apt, at least in the beginning, to show different features. The nodular type appears to me to be transmitted by an arthropod capable of burrowing into the skin, possibly the *Demodex folliculorum*, as suggested by Borrel. The maculo-anaesthetic type may possibly be due to direct contact infection.

I may mention that Wherry found acid-fast bacilli in the lice of leprosy rats. However, this is by itself not sufficient evidence to incriminate Syphonaptera as disease carriers in this connexion. Leprosy bacilli can be found in the blood of lepers during the rises of temperature that occur frequently during the course of the disease. At other times, if the blood is taken from a spot free from nodules, none are to be found. This fact may possibly explain why Donald Currie did not find any acid-fast bacilli in the 493 specimens of *Culex cubensis* he examined thirty minutes to three hours after they had fed upon leprosy patients.

Turning now to the cases which have contracted leprosy without ever having left England or a country where leprosy is unknown, may we not suggest that by some extremely fortuitous circumstance they may have been stung by some arthropod ectoparasite off a leprosy rat? The sarcoptes common to rodents will attack man, according to Brumpt.

These suggestions are simply put forward with the

intention of collecting independent observations which may prove or disprove my views. If the germs I have demonstrated to-day are the causal agents of leprosy in man and rats, we may hope in time to have experimental proof of many of the points which up till now it was impossible to clear up in connexion with leprosy.

I am quite aware of having given but scanty details regarding several of my experiments; in fact, I have had to pass over several observations because the time for reading a paper is strictly limited. I am, however, preparing a report for the London School of Tropical Medicine, which I hope to be able to publish, and which contains a full consideration of the literature on the subject.

My conclusions are :

1. It is not sufficient to culture an acid-fast or other organism from one or several cases of leprosy to enable a statement to be made as to its relationship to the disease. This must be the result of complete animal experiments and extensive serological tests.

2. It is possible to culture from cases of human leprosy an acid-resisting diphtheroid which acquires acid-fast properties on being injected into mice or rats. It is possible to regain the germ by culture from the organs of the animals infected as an acid-fast rod. Reinjecting into mice or rats, it causes the identical changes of genuine spontaneous rat leprosy, and very striking analoga of the glands and organs of human cases.

3. No special medium can guarantee a culture from a case of leprosy. The growth depends upon circumstances which we do not so far know and cannot control. This applies to the *B. leprae* in its acid-fast shape, as seen in lepromata. The most favourable media appear to be either placental-extract-agar or horse-serum-nutrose-agar with the addition of 2 per cent. ground-up smegma bacilli.

4. Kedrowsky's work on the variable morphology and staining properties of the lepra bacillus appears to be confirmed.

5. Agglutination, precipitation, complement-deviation, and percutaneous tests can be used to prove the relationship of acid-fast or other germs cultured from cases of leprosy.

6. Rat and human leprosy appear to be identical diseases; it is therefore possible that the germ of both can be transmitted from one to the other, given an appropriate intermediary. As rat leprosy appears to be endemic in England, this may be an explanation of the sporadic cases in people who have never been abroad.

These investigations were carried out in the Lister Institute of Preventive Medicine and at the London School of Tropical Medicine.

ON THE TRANSMISSION OF LEPROSY TO ANIMALS BY DIRECT INOCULATION.

By H. BAYON, M.D..

BEIT MEMORIAL RESEARCH FELLOW.

(From the Lister Institute of Preventive Medicine.)

APPARENTLY there exists the widespread opinion that leprosy has never been transmitted to animals by means of direct inoculation of nodules from the human being. In fact, Abraham, in his careful and comprehensive chapter on leprosy in Allbutt and Rolleston's *System of Medicine*, after reviewing the various attempts to inoculate animals, comes to the conclusion that

The evidence given above, though to some extent conflicting, may on the whole be regarded as supporting the view expressed by Besnier—that leprosy is strictly a human disease which cannot be transmitted to animals.

I need not refer to the similar views held for many years regarding syphilis, which, being a disease due to a protozoon-like germ, might possibly be specific for the human race, as appears to be the case with malaria in man. And yet, after Bertarelli and Parodi succeeded with their first experiments, no one seems to have found it impossible to transmit syphilis to the rabbit.

Leprosy is a disease due to a definite bacterium, and there appears to be no special reason why the rabbit or any other laboratory animal should not be inoculable with it. The existence of spontaneous rat-lepra affords, in fact, the best ground for postulating the possibility of transmitting lepra to animals.

However, in a matter like this it is useless to quibble with words about possibilities. All arguments can be swept aside by the statement of successful inoculation, supported of course by detailed histological evidence.

I am not taking into consideration the infection of animals with leprosy after cultivation of the germ.

Rost has reported that he has produced the clinical features of leprosy in a monkey by injecting his streptothrix.

Duval, by injecting his strain of *B. leprae* in enormous quantities into a monkey, produced lesions which, he says, could not be distinguished from those in the human being. Kedrowsky has produced in mice and rabbits lesions

extremely similar to those of human leprosy by injecting the germs he cultivated from lepers. I have had an opportunity of examining his microscopical slides personally, and admit that they are most convincing.

With a diphtheroid-like germ cultivated from a leper I have produced lesions in a mouse indistinguishable from those occurring in spontaneous rat leprosy.

Now, however, I am concerned with reviewing the work done by grafting lepromata to animals direct from the patient. So far as I can gather from the literature on this subject the positive results which have been recorded are as follows:

1881. Neisser, after twenty-four unsuccessful attempts on rabbits, injected two dogs with leprosy nodules, and concluded from the results that leprosy developed locally at the site of inoculation.

1883. Damsch grafted lepromata in the anterior eye chamber of two rabbits. The animals died after 139 and 219 days. Apparently a slight increase in the quantity of acid-fast germs had taken place. He also inoculated two cats in the abdomen with leprosy nodules. After 120 days numerous bacilli were found in the newly formed tissue round the atrophied nodules.

1888. Vossius carried out experiments in a similar fashion to Damsch on rabbits and had identical results. He also considered that the acid-fast germs had multiplied.

1885-6. Melcher and Ortmann inoculated four rabbits in the same way. They succeeded in getting, after four to ten months, definite metastases and deposits of acid-fast germs in the spleen, liver, caecum, pleura, and pericardium. They stained these germs with Baumgarten's differential stain and found that they had the staining properties of Hansen's germ.

1887. Wesener injected several rabbits with leprosy nodules, and in two animals out of eight, after periods of four and a half months and eight months respectively, he found lesions apparently identical with those described by Melcher and Ortmann—that is, nodules from the size of a pinhead to that of a pea in the lungs, on the pleura, on the epiploon, in the liver, in the lymphatic glands, spleen, kidneys, caecum, and peritoneal surface. Caseation and giant cells were present. In the eyes of the rabbits he obtained after six and eight months a congregation of round cells choked up with acid-fast germs. He considered that the generalized lesions were due to tuberculosis and that the ocular lesions were caused by dead bacilli, because acid-fast germs can be demonstrated in the eye of the rabbit after inoculation with nodules which have been kept for years in alcohol.

1901. Barannikow confirmed these results on one rabbit.

1893. Wnouwow inoculated twenty rabbits with leprosy nodules in various fashions (intraocularly, subcutaneously, intraperitoneally). In fourteen rabbits he got lesions which he considered to be of tuberculous nature. No

confirmatory tests with guinea-pigs appear to have been made.

1893. Tedeschi inoculated a leproma into the dura of a monkey. The animal died after eight days; that is far too brief a period for any conclusions to be drawn.

1902. Ivanow injected several guinea-pigs with leprous nodules. In one case the animal was killed after eight months and found to have nodules in the epiploon, which Ivanow considered were due to multiplication of the bacilli.

1905. Thiroux inoculated five rabbits with leprous material. The animals lived thirteen to twenty months and presented various lesions at autopsy, which Thiroux considered to be tuberculous.

1906. Nicolle inoculated monkeys with ground-up nodules and succeeded in getting localized lesions.

1909. Marchoux and Borret inoculated a chimpanzee under the skin of the ear with a freshly-excised leprous nodule. It apparently increased in size during three months and then began to be resorbed. An examination of the blood showed a few leucocytes with badly-staining bacilli. The animal died ninety-six days after inoculation. The nodule had reached the size of a split pea, and under the microscope was shown to consist of three layers. In the middle were the remains of the original tissue, quite necrosed, with a few loose acid-fast germs. Around this was a considerable number of lymphocytes, enclosing numerous bacilli, and at the periphery organized connective tissue with a few cells filled with bacilli. The authors considered that a proliferation of the original germs had taken place.

1909. Sugai inoculated Japanese dancing mice intra-peritoneally with an emulsion of fresh leprous nodules and found as a result the development of miliary granulomata on the peritoneal lining, especially in the hepatic region and epiploon. Also the bronchial and peritoneal glands showed characteristic leprous lesions. All contained acid-fast germs. The attempt to transmit the disease from these to other mice failed.

1909. Kitasato appears to have successfully inoculated an orang-outang on the cornea. Only scanty details were, however, given.

1909. Stanziale reports upon some very careful and thorough experiments on rabbits. In one typical case he inoculated into the eye of the animal a fragment of leproma. During the first few days a marked hyperaemia developed round the opening in the cornea. Soon afterwards a constant diminution in the size of the nodule could be made out, accompanied by the formation of an opalescent exudation, which took its departure from the inoculated piece of tissue and spread out as a fan-shaped segment. After twenty days the nodule became stationary and remained so during fifteen to twenty days without any noticeable change taking place. After forty days the nodule began slowly to increase till it had tripled its original size. In the same time small grey nodules made

their appearance and could clearly be distinguished on the surface of the inoculated tissue. These little nodules attained the size of a pin's head. Some appeared to be adherent while others were loose and independent. After the initial hyperaemia due to the operation had disappeared, a new formation of blood vessels took place, which spun a delicate network round the piece of tissue inoculated. After seventy days the rabbit's eye was enucleated, part of the tissue was used for histological observations and the rest was grafted into the cornea of two other rabbits. In one the graft got resorbed without any further results in the course of two months. In the other rabbit the graft began to increase in size after fifty days and produced similar changes to those described in the foregoing animal. The experiments are being continued by the author, but to date the results obtained are: thirty-one rabbits have been inoculated and in eight it was possible to produce leprous ocular lesions. In some of the positive cases the material was taken eighteen to nineteen hours after excision from the patient. In all cases in which the inoculation succeeded the Wassermann reaction was positive. As a control Stanziale inoculated lepra nodules into the abdomen of another rabbit and found at intervals after a month the reaction constantly negative. The same negative result was achieved after grafting into the cornea of rabbits pieces of human skin or dead leprous nodules.

These experiments of Stanziale have been most carefully carried out, and full descriptive details are given.

1911. Duval inoculated a series of animals—namely, four rats, four white mice, and four Japanese dancing mice—with grumous material taken from an acute case of human leprosy which had developed numerous soft subcutaneous leprous masses and had repeated attacks of leprous fever. A small quantity of material was used in each case—namely, 0.5 c.cm. emulsified in 1 c.cm. of normal saline solution. Some were injected intraperitoneally, others subcutaneously.

The two white mice which received intraperitoneal injections died fourteen days after the inoculation. At the autopsy both showed a general infection of the peritoneum with a pure growth of the *B. leprae*, while the mesentery, omentum, visceral and parietal peritoneum contained numerous minute, firm greyish-white nodules, which on microscopic section proved to be typical leprous lesions. The most surprising feature was the occurrence of a slightly turbid, semigelatinous exudate, which microscopically consisted almost entirely of large mononucleated cells (macrophages). Great numbers of these cells were filled with acid-fast bacilli, and scarcely any were found that did not contain a few. No remarks are made about the other animals.

1911. Couret fed two goldfish on teased nodules from a leper. No other food was allowed the animals until they had disposed of the leprosy tissue, after which they were cleansed and transferred to a clean aquarium.

The first was examined twenty-four days later; acid-fast organisms had not been found in the faeces for three days before the animal was killed. No evidence of infection was apparent externally or internally. Films made from the omentum and other organs showed a few scattered acid-fast bacilli. The average number of bacilli to the slide was about four, and no change in morphology was seen. The second fish was examined after thirty-seven days. The findings were similar to those in the first one. With reference to these experiments it should not be forgotten that tap water appears to harbour acid-fast germs frequently, as shown by Brehm, Beitzke, Schern und Dold. The tap water at the Lister Institute also contains acid-fast germs.

My own experiments on this question are as follows:

I injected four rats on two occasions in the testes with ground-up nodules from a case of leprosy from Mauritius. Two rats did not show any macroscopic lesions, even after four months. One rat, however, developed, four weeks afterwards, a nodule at the site of inoculation, which grew to the size of a small pea. On puncture it showed acid-fast germs and necrosed tissue, some of which I do not doubt represented the original cells injected. After five weeks the rat died,* and I injected the whole of the nodule subcutaneously into two other young rats. Quite lately, that is, three months after inoculation, one of the rats is losing fur on the surface of its abdomen, without there being any signs of the usual rat scabies about the ears or tail. Small shotty nodules are felt under the skin.

Another rat developed now, three months after the second injection, a nodule in the left testis. This broke down, and was found to contain acid-fast rods in great quantity. I cannot decide so far if a multiplication of the lepra germs has taken place. I do not intend admitting a multiplication till definite metastasis can be found in other organs, and not only germs at the site of injection.

However, taking into careful consideration the literature I have quoted, I come to the conclusion that leprosy is directly transmissible to rats and rabbits from the human being; that, however, a very long time of incubation must be allowed for, and very many unsuccessful attempts and partial results are to be expected, as has been the case in my experiments.

I consider the results of Melcher and Ortmann, Wesener, Wnukow, Stanziale to have been successful. Spontaneous tuberculosis in laboratory animals is extremely rare. During ten years I have not seen a single case, and the experience of other workers I have asked is similar to mine.

It is quite true that lepra "bacilli" are so quiescent, and cause such minimal reactions in the tissues they invade, that it is not possible to distinguish whether they are dead or alive, the word "alive" being taken in the sense of

* I can add that no acid-fast germs were found in the organs of this rat. Microscopical sections were made of liver, spleen, and testes. The disease, if transmitted, was localized.

"capable of multiplying." We have no other method of deciding whether an acid-fast germ is living or killed but that of testing its capacity for multiplication on a laboratory culture medium. And this has been for many years a controversial point with leprosy, because no culture so far has succeeded in reproducing anything like leprous lesions in the animals experimented upon. Lately, however, Kedrowsky has published drawings and photographs of microscopical sections which leave little doubt that he has succeeded in producing in rabbits and mice lesions indistinguishable from those occurring in leprosy of the human being. I have now been working with this germ for over a year and have not been able to find any evidence that did not speak in favour of its being leprosy.[†] This germ produces in the liver of rabbits and in guinea-pigs lesions which, taken singly and separately from the others, might be mistaken with the chronic type of tuberculosis which occurs in rabbits and very exceptionally in guinea-pigs, and which can easily be imitated by the intravenous injection of an avian strain into rabbits. This appears to me to also be circumstantial evidence in favour of leprosy having been inoculated on several occasions to rabbits. The lesions were wrongly taken to be tuberculosis.

With regard to Campana's and Wesener's experiments, we must bear in mind that in the analogous disease in rats, genuine or spontaneous rat leprosy, we also find the incubation extremely long, up to seven months, and one is often in doubt whether the bacteria found in the lesions have multiplied or are the original ones injected. And yet there is not much doubt possible that in many cases we succeed in transmitting the disease by inoculation through several generations. My conclusions, therefore, are that:

1. Leprosy can under certain circumstances be transmitted to animals, the main difficulties being the high percentage of unsuccesses and the long incubation.

2. The most suitable animals appear to be the rabbit, rat, or mouse. In the rabbit the intraocular method is preferable. The resulting lesions are often not visible to the naked eye, and are similar to those caused by certain strains of human tubercle.

3. In the rat the incubation is also very long, the resulting lesions comparable to those occurring in spontaneous leprosy of the rat.

4. Not all leprous patients' nodules are equally "infective" for animals. In some cases the lepra "bacilli" appear to be absolutely incapable of multiplication, for reasons we do not at present know.

5. According to Stanziale's experiments, the "Wassermann reaction" is positive in rabbits which have successfully been inoculated.

[†] I cannot in this paper describe the serological and experimental tests I have carried out with Kedrowsky's germ. It is an actinomyces-like bacterium, and in its acid-fast stage morphologically identical with the "bacilli" found in the serum and pus of lepers.

THE CULTURE AND IDENTIFICATION OF THE GERM
OF LEPROSY AND THE RELATIONSHIP OF THE
HUMAN DISEASE TO RAT LEPROSY.

BY H. BAYON,
Beit Memorial Research Fellow.

(Paper read at a Meeting of the Society of Tropical Medicine and
Hygiene, Friday, 16th February, 1912).

You are asked to decide this evening, as far as is possible in the short time at our disposal, whether the statements I shall make are justified or not. They are:—

(a)—The germ of leprosy can be cultivated from the lesions it causes in the human being.

(b)—Leprosy can be transmitted to animals, either after the culture of the germ or direct from the human being.

(c)—Rat leprosy and human leprosy are closely allied diseases; in fact the germ of both may be identical.

I intend to lay the whole weight of my arguments on the slides and microscopical sections being demonstrated. I therefore am unable to consider this evening much of the work done by others in the same field. I certainly shall do so in my next paper.*

The first projection shows the non-acid-fast and non-acid-resisting germ I cultivated from the nodules of a case of leprosy at the London School of Tropical Medicine. It is morphologically identical to the germ that KEDROWSKY got from his cases in Russia. You will note the peculiar way in which it has grouped itself round the original "bacilli." In other parts of the field it showed definite branching forms. It may be called an actinomycotic germ or a streptothrix, according to taste. It grew rapidly as a white viscid growth on placental-extract-glycerine-agar, and presented morphologically the peculiarities of a contaminating

* Results of cultures of the *B. Lepre* have been recorded by the following:—

1882, GAUCHER ET HILLAIRET, HANSEN. 1886, NEISSER. 1888, BORDONI-UFFREDUZZI. 1889, BABES, GIANTURCO. 1890, STALLARD. 1891, KANTHACK, RAKE and BUCKMASTER. 1892, ABRAHAM, CAMPANA, DUCREY. 1897, E. LEVY, CZAPLEWSKY, STICKER and DIEUDONNÉ. 1898, SPRONK, TEICH. 1899, CARRASQUILLA, BARANNIKOW. 1901, KEDROWSKY, F. LEVY. 1902, VAN HOUTUN. 1903, GUEBERT, KLITIN, KARLINSKY. 1904, ROST, ZANONI. 1905, DEYKE and RESCHAD, WEILL. 1909, SHIGA, CLEGG, SERRA. 1910, DUVAL, ROST, WILLIAMS, TWORT. 1911, BAYON, BOECK. Unpublished results, FREMLIN (1904). HEWLETT (1910).

germ from the air. In my first paper on mentioning it I said that I feared it might not have anything to do with leprosy. The experiments I have since carried out with it have made me necessarily change my opinion. The stain used is ZIEHL-NEELEN, the magnification is 1,000 diameters. (Fig 1).

The second slide shows the pleomorphic, diphtheroid-like organism cultivated on two different occasions from the nodules of the same case, also on placental-extract-glycerine-agar. Note its apposition to the original acid-fast "bacilli." This germ is acid-resisting—*i.e.*, after staining for ten minutes or more with warm carbol-fuchsine, it will resist the bleaching effects of 2 per cent. sulphuric acid for five seconds and not be stained by following treatment with dilute (1/10) Loeffler's methylene blue, but some of the germs will show then a clear ruby-red colour, and many others will be of a fainter red hue. Professor MICH, who is present this evening, will, I hope, explain the cause of this stain reaction, from the standpoint of the presence of "nastin" in acid-fast germs. Note the branching forms and the clubbed shapes. This slide is also stained according to ZIEHL-NEELEN, therefore the germs are blue. Magnification: 1,300 diameters. (Fig. 2).

The third projection is a coloured photo, according to Sanger-Sheppard's method, which has been very kindly taken for me by Messrs. Leitz. The magnification is 1,100 diameters, and I am sure you will admire the sharpness of the contours and the absolute precision of the colouring. This is the third or acid-fast stage of the germ, as it can be seen in the nodules of the leprosy patients. Note the slightly thickened bacilli, identical in their morphology to those seen in the pus of leprosy. (Fig. 3).

The germ at this stage is absolutely acid-fast—*i.e.*, after staining for three minutes with warm carbol-fuchsine it cannot be bleached even with 20 per cent. nitric acid in three minutes time, and will not stain blue after treatment with Loeffler's alkaline methylene blue. I must, however, remark that not all germs in a slide are equally acid-proof. This is, however, an observation that can also be made on cultures of any germ in its acid-fast stage. Incidentally I may mention that I do not consider that the property of being acid-proof sufficiently characterises any germ, as it is simply the appearance of a transitory condition in many cases I have investigated. Probably the same holds good for many more

I have not examined from this point of view, which however has already been most ably put forward by FOULERTON on various occasions.

What are my proofs that these three different conditions represent but the successive stages of HANSEN'S "bacillus," which is originally a filamentary branching, non-acid-proof organism?

First of all I must mention the work of KEDROWSKY, who, in 1901, expressed the opinion, based on experiments, that leprosy might be due to an organism possessing a developmental cycle similar to certain actinomycotic germs. Later on we have DEYCKE'S, ROST and WILLIAMS' work on the subject: moreover, HERMAN'S and ABRAHAM'S observation on the incomplete acid-fastness of HANSEN'S "bacillus" in young nodules.

Next I should refer to the experiments of SANFELICE, LOMBARDO-PELLEGRINO, CZAPLEWSKY, GRASBERGER, TOBLER, AUJETZKY, WEBER, NABARRO (these last as yet unpublished) on the varying degree of acid-fastness of various germs according to the media they have been cultivated upon or after passage through animals.

I should also mention the observations of BIRT and LEISHMAN, FLEXNER, KEDROWSKY, RABINOWITSCH, SILBERSCHMIDT, NOCARD, BEREST-NEW, MOELLER, EPPINGER, SABRAZES ET RIVIÈRE, who have described acid-fast and partially acid-fast branching germs, in other words actinomycotic germs, capable of breaking up into acid-fast rods, from various sources.

As regards the diphtheroid stage we have GORDON'S study of a streptothrix which broke up into diphtheroids and which was cultivated from the throat of a child. Moreover, branching forms in diphtheroids have been seen by KLEIN, FRENKEL (1895), BABES (1895), BERNHEIM and FOLGER (1896), SCHIWAN (1900). SPIRIG has on two occasions even got hyphæ in diphtheroids—an observation which I admit has not been generally accepted, though the fact is certainly not out of the realm of possibility.

Branching in tubercle has been repeatedly recorded: by ROUX ET NOCARD (1887), METSCHNIKOFF (1888), KLEIN (1890), MAFFUCCI (1892), FISCHER (1893), BABES (1895), STRAUSS (1895), SCHIWAN (1900).

To persuade you, if necessary, that acid-resisting branching germs do exist, I will project a coloured micro-photograph of BIRT and LEISHMAN'S pathogenic streptothrix, stained according to ZIEHL-NEELENSEN. This photo has been made by Dr. REID, of the Lister Institute, whom I

wish to thank for the trouble he has taken in the preparation of these lantern slides.

I am obliged, however, to keep myself to my own personal observations, and so I will project a colour-photo of the results I got by injecting the non-acid-proof branching germ into the peritoneal cavity of a rat. Three rabbits and one rat were injected. The rabbits died before any definite change could be observed, the rat developed a lump at the site of injection. This lump was punctured three months after inoculation and the juice showed leucocytes crammed with definitely acid-fast germs, which still had retained their "ray-fungus" habitus. I took a culture and that was the acid-fast germ I demonstrated as my third slide. The non-acid-fast germ had turned into an acid-fast "bacillus," and could now be cultivated as such. This culture is very similar to KEDROWSKY'S strain, because it is a white, slow growing, peculiarly wrinkled organism, not at all unlike to avian tubercle.

I also injected the diphtheroid intraperitoneally into a mouse. After three weeks the mouse died and I was able to regain the germ as an acid-fast diphtheroid in sections and cultures. The culture was, however, contaminated by cocci and died out. The corresponding slides are under the microscopes. The lesions resulting in the peritoneal glands of the mouse were absolutely identical to those seen in spontaneous rat leprosy, as regards staining reaction, position and quantity of "bacilli."

My next projection relates to the lesions resulting from the injection of the germ in its acid-fast stage. This is the spleen of a mouse, seven months after it had been injected intra-peritoneally with 0.3 of an emulsion of KEDROWSKY'S strain. The liver also showed similar lesions. These enormous red patches consist all of acid-fast bacilli, which have hardly given rise to any inflammatory reaction, showing, therefore, their low toxicity. You will admit that the analogy with human and rat leprosy is most striking. (Fig. 6).

This is a section from the lung of the same animal. Note the absence of caseation, the minimum inflammatory re-action, the non-appearance of giant cells of the LANGHAN'S type, the absence of sclerosed vessels, all details which help to distinguish HANSEN'S "bacillus" from its near relation, the tubercle. These slides should prove that a non-acid-resisting branching germ is capable of acquiring acid-proof properties after injection into animals, that it can then be regained by culture

as an acid-fast rod, and that it can cause lesions extremely similar to those occurring in leprosy of the man and rat. Whether this germ is HANSEN's "bacillus" or not, I shall prove by other independent methods.

So far my work had but been the confirmation of the results of KEDROWSKY. I thereupon undertook to continue the work to a definite conclusion. Acid-fast germs are absolutely ubiquitous, they have been found practically everywhere where they have been carefully looked for, in dung, in earth, in tap-water. I am demonstrating one of these tap-water acid-fast taken from the tap in my room at the Lister Institute. In the same water I encountered amœbæ of the verrucosa type, which contained acid-fast, as also did, but to a much lesser extent, the *Euplotes* sp. which swarmed in the mould growing round the tap.

On the table you see racks with over forty different strains of these acid-fast, the great majority of which have been found in milk. Fourteen of these germs I owe to the kindness of Dr. NABARRO, who has isolated them himself.

You see, therefore, that it is absolutely necessary to identify any acid-fast or other germ cultivated from one or several cases of leprosy before stating its relationship to HANSEN's "bacillus." Professor KEDROWSKY having kindly put one of his strains at my disposal, I set to work distinguishing it from the three germs it might be, viz.: human tubercle, smegma or avian tuberculosis. I determined to find a series of tests that would enable me to identify any germ I might get and to definitely state whether it had anything to do with HANSEN's "bacillus" or not.

By means of intravenous injection of the common smegma "bacillus" I produced the clinical features of leprosy in the rabbit, as described in previous papers of mine. The nodules in the skin, in the kidney, showed enormous felt-like masses of acid-fast germs which you see in this section. There is sufficient resemblance to the lesions produced by HANSEN's "bacillus" to enable an unskilled or even enthusiastic worker to claim he had produced the identical lesions of leprosy by the injection of some common saprophytic acid-fast, such as the smegma germ. (Fig. 5).

However, we know that the germ of leprosy belongs to the "tubercle" group, as has been confirmed (after the histological evidence had given several valid reasons for this view) by means of serological tests by MUCH and HÆSSLER, and also WILLIS. On injecting KEDROWSKY's

strain intravenously into rabbits, I soon found that it caused lesions extremely similar to those caused by the intravenous injection of the avian tubercle into the same animal. You will notice the similarity caused by the "ray-fungus" like disposition of the organisms (which, however, can also be produced by the human variety of tubercle and by the timothy grass acid-fast germ [SCHULZE, LUBARSCH, ABBOT and GILDERSLEEVE]) in the two slides I am projecting.

My first step was, therefore, to exclude that the strain was tubercle, human or avian. I soon found that it was non-pathogenic for guinea-pigs, and that fowls could be injected with half a slope without showing any lesions on autopsy two months afterwards. It was absolutely necessary to carry out these experiments because under circumstances even human tubercle is capable of causing on injection into rabbits, lesions very similar to leprosy.

This is a specimen demonstrating the enormous quantity of bacilli which have accumulated in a nodule in the cœcum of a rabbit, which was injected intra-peritoneally with a full slope of human tubercle "bacilli," which had been heated for an hour at 60° C. You will see that the whole field is red with them. The similarity to leprosy is striking, but the germs are much more scattered about than is the case in leprosy, where the "bacilli" are more apt to congregate in groups.

Having persuaded myself that the germ cultivated from lepers was not an avian or other tubercle, as its appearance and lesions it caused in rabbits might well lead one to believe, I determined to test the serum of lepers against the acid-fast stage of this organism by means of the complement fixation method.

I prepared my antigen by alternately freezing and rapidly thawing an emulsion of "bacilli" in distilled water, with the intent of bursting, if possible, the waxy capsule which appears to envelop most acid-fast germs.

In each complement absorption test I put up at least four rows of tubes and never tested a germ by itself, but always in the same time as some other closely allied acid-fast germ. This method has been most successfully made use of by H. R. DEAN in distinguishing various allied strains of the para-typhoid group. In each row I diluted my serum 1/5, 1/10, 1/20, 1/40, 1/80, and so forth. In the first row, however, I made use of the antigen in full dose, in the second I linked up the serum with

half doses of antigen, in the third with quarter and in the fourth with one-eighth of the quantity used in the first row, the dilution being carried out with saline.

I made several experiments which enabled me to distinguish quite clearly avian from human tubercle with appropriate sera. However, here I am only concerned with giving my results as regards leprosy serum.

These are the results obtained by linking the serum of a leper from Mauritius with KEDROWSKY'S and DUVAL'S strain of leprosy.

In the first row KEDROWSKY'S strain reacted up to $1/160$.

In the second row up to $1/40$ to $1/80$.

In the third row $1/20$ to $1/40$.

In the fourth row $1/10$ to $1/20$.

DUVAL'S germ gave following results, viz :—

In the first row it reached up to one in $1/5$ to $1/10$.

In the second row slightly $1/5$. All others negative.

In another experiment I tried KEDROWSKY'S and ROST'S strain against one other, using the serum of a patient, for which I have to thank Sir ARTHUR DOWNES and Dr. ABRAHAM. These are the results in one case :—

Dilution of serum $1/8$, $1/16$, $1/32$, etc.

KEDROWSKY'S germ.

In the first row reacted till $1/64$.

In the second row $1/32$.

In the third row $1/16$. The same in the fourth row.

ROST'S germ.

Dilutions of serum $1/7$, $1/14$, $1/28$, etc.

In the first row $1/7$ to $1/14$.

In the second $1/7$. All others negative.

I will mention that results agreeing with these, as far as the specificity of KEDROWSKY'S germ is concerned, have been arrived at by Dr. HUNT, at St. George's, by means of the opsonic index test. Dr. LEDINGHAM found a very high tropin-content against KEDROWSKY'S germ in the inactivated serum of a patient who had received several injections of bacterial extract of KEDROWSKY'S strain of leprosy.

These experiments, taken together with the slides I have shown you ought to warrant the claim that the germ has been identified, and that if it had been a contamination of some sort, it would not have fulfilled

all the conditions it has done so far, including that of giving a tuberculin-like reaction on injection as a bacterial extract made on the lines of KOCH's Alt-tuberkulin. I cannot, however, give too much value to this test by itself, as it is not sufficiently specific to my mind.

The conclusion that I therefore draw is that KEDROWSKY's germ is indetical with HANSEN's "bacillus" because it has repeatedly been cultivated from lepers, because it causes leprosy lesions in rats and mice, and last, but by no means least, because it reacts specifically with the serum of lepers in a way that neither tubercle or avian do. Moreover it is not identical with any other known germ.

My culture is morphologically identical, to the naked eye and under the microscope, to this strain. It grows at the same rate, in the same fashion on the same media, therefore I take it it is also leprosy, though I have not been able, so far, to put it through all the tests necessary for its identification.

The next question to solve is whether leprosy can be transmitted to the common laboratory animals, the rabbit, rat, mouse, direct from the human being, without first culturing the germ. I have published a paper on the subject in the *British Medical Journal* of February 24th, and do not wish now to go over the same ground again. I will simply project a photograph of the results obtained in rats and can only refer to the slides under the microscope. Regarding rabbits I will remark that the similarity of the resulting lesions to those of tuberculosis, made them not be recognised as leprosy and gave rise to the view that the authors who had published positive results had sown leprosy and reaped tuberculosis. There can now not be much doubt that the results of MELCHER and ORTMANN, WNOUKOW, WESENER, STANZIALE, and possibly also THIROUX were true leprosy. In all these animal experiments we must, however, prepare ourselves to have long series of incomplete or negative results.

Now to the question of rat leprosy. It is well known that the last leprosy conference after discussing the matter at length came to the conclusion that the human and rat disease had nothing to do with each other.

In my very first paper on leprosy I stated that I considered both diseases to be either identical or closely allied as human is to bovine tuberculosis because :—

(a)—The lesions in both diseases are microscopically identical (Fig. 4).

(b)—Because GEORGE DEAN found that:—

(1) The serum from a case of human leprosy agglutinated the acid-fast bacillus from the rat. Normal human serum had no agglutinating power.

(2) The serum of rats inoculated with the acid-fast bacilli agglutinated the diphtheroid DEAN cultivated from leprosy rats. Normal rats' serum did not agglutinate the diphtheroid.

(3) Normal human serum and the serum from a tuberculous patient failed to agglutinate the diphtheroid, whereas the serum from a case of leprosy had distinct agglutinating properties.

To this evidence I am able this evening to add a further argument in favour of both diseases being very closely related. We have succeeded in cultivating from a case of rat leprosy, an acid-fast germ very similar to the one got from human leprosy.

Dr. LEDINGHAM having kindly got me some nodules from leprosy rats, found in the course of plague investigations, I inoculated the ground up material, after treating it for a short time with 1/20 antiformin, intravenously into a rabbit and intra-peritoneally into a rat. The rabbit is still alive, five months after injection, but reacts in a complement absorption test up to 1/60 with human leprosy antigen.

The rat was killed one month after inoculation and showed the naked eye changes in the epiploon, *i.e.*, nodules, which have been described by DEAN in the disease. With the help of Dr. MARTINEZ, cultures were attempted on several tubes of fish-juice-glycerine-agar. Only one culture was successful, out of a dozen attempted and that was with juice from the spleen. After a month incubation at 37 C., small pinhead size, scattered ivory-beaded colonies appeared. These consisted of acid-fast rods. They were sub-cultured and you see the result on the table: a white wrinkled growth, very similar to human leprosy and therefore also not unlike to avian tuberculosis. Under the microscope they are short plump rods, absolutely acid-fast.

I am going to identify the germ in course, but in view of the various specimens I have shown this evening I think I am justified in considering it to be very probably rat leprosy. The microscopical appearances in the liver, spleen, omental nodules from the rat from which the culture was taken tended to show that a multiplication of the germs had taken place, however, not to a very marked extent.

What are now the conclusions to be drawn from a clinical stand-point of view in relation to the observations I have recorded? I believe that my experiments entitle me to say that to all appearances the acid-fast stage of HANSEN'S "bacillus" is very slightly infective, because you have to inject relatively heavy doses to get definite, progressive lesions. On the other hand, the non-acid-proof conditions are more apt to cause the disease, even if the incubation period may be very long.

This leads me up to state that I believe that the early and progressive stages of leprosy are the only ones that are dangerous from an infective point of view.

How does this agree with clinical observations?

Though very slow in multiplying and exceedingly quiescent, the acid-fast "bacilli" are capable of resisting, at least as far as their morphology is concerned, thermic, chemical and physical influences to a very considerable extent. Under the microscope you will see some morphologically intact "bacilli" which have been boiled in ether for six hours at a stretch. I can show similar slides after drying, grinding and then boiling for twenty-four hours. This explains the fact that we never have any proof whether the "bacilli" in a lesion are capable of multiplying or not till we have definitely seen them do so in an animal or on media. Morphologically intact, faultlessly staining bacilli in a leprous nodule may be dead as an autoclaved culture, though of course death and life in relation to germs are certainly not such hard-and-fast distinctions as is the case with vertebrates.

I believe that an organism incapable of multiplying may still be able to eliminate toxins and in determining this exact point, we may open up the possibility for therapeutic treatment.

The analogy, if not identity of human and rat lepra, ought to enable us to throw light upon the way leprosy is transmitted, and therefore put us in condition to tackle the scourge amongst human beings from the standpoint of prevention.

These investigations were begun at the London School of Tropical Medicine, and then continued at the Lister Institute, and I desire to express my indebtedness to Sir PATRICK MANSON and Dr. MARTIN, and the Staff of both Institutions for the help and advice they have given me.

ILLUSTRATIONS.

Fig. 1.—Culture on horse-serum-nutrose-agar, from nodules of case of leprosy at the London School of Tropical Medicine. Stain: ZIEHL-NEESEN. Magnification: 1,000 diameters. Drawing by M. RHODES.

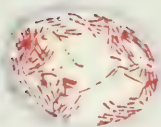
Fig. 2.—Diphtheroid-like pleomorphic organism from same case. Third sub-culture one month on placental-extract-glycerine-agar. Possibly a slight multiplication of the acid-fast bacilli may have taken place. Stain: ZIEHL-NEESEN. Magnification: 1,300 diameters. Composite drawing from various parts of slides by M. RHODES.

Fig. 3.—Last or acid-fast stage of the actinomycotic germ of leprosy. Pure culture, two months' growth on fish-juice-glycerine-agar. Stain: ZIEHL-NEESEN. Magnification: 1,100 diameters. From a coloured photo according to SANGER-SHEPPARD's method, by Messrs. E. LEITZ.

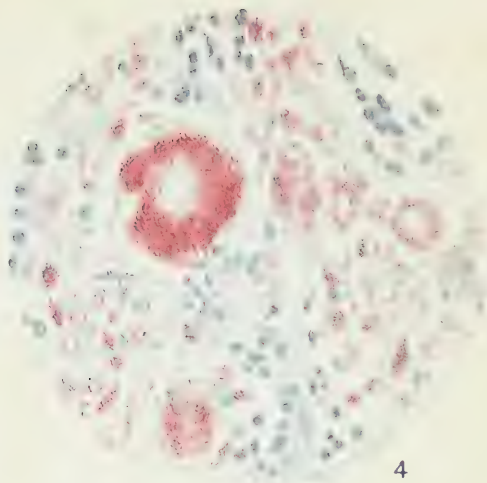
Fig. 4.—Section of omental nodule from leprosy rat, found in Ipswich by Drs. PETRI and MACALISTER. Stained according to ZIEHL-NEESEN. Magnification: 500 diameters. Drawing by M. RHODES.

Fig. 5.—Lesions produced in kidney of rabbit by intravenous injection of MOELLER's smegma germ. Stained according to ZIEHL-NEESEN. Magnification: 300 diameters. Drawing by M. RHODES.

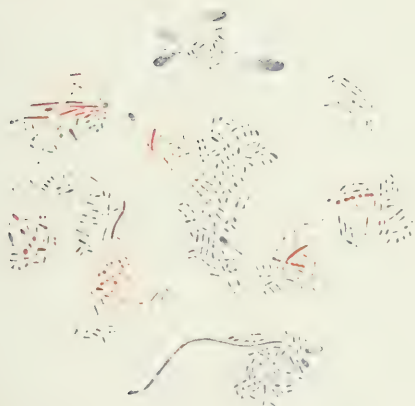
Fig. 6.—Lesions produced in spleen of mouse after intra-peritoneal injection of acid-fast stage of human leprosy germ. Stained according to GRAM. Purple masses consist of acid-fast, gram-positive bacilli. Magnification: 400 diameters. Colour photo according to SANGER-SHEPPARD, by Messrs. E. LEITZ.



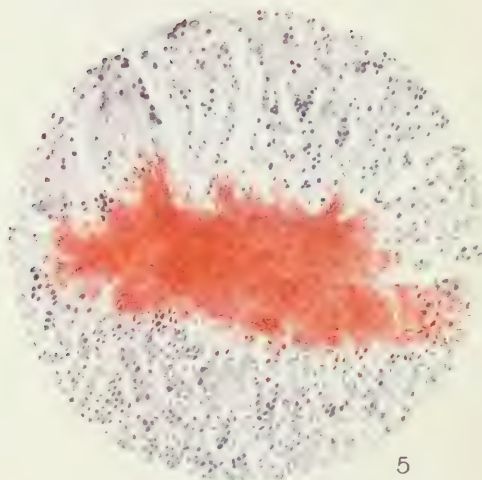
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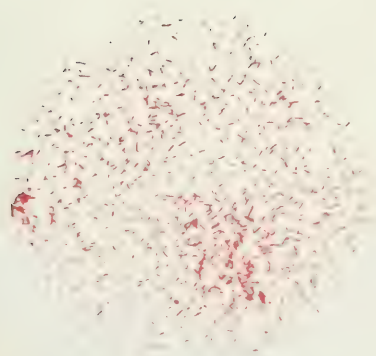
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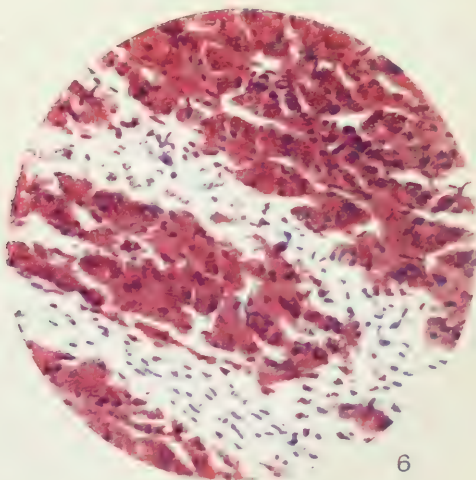
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6

On the Factors Concerned in Agglutination.

By H. R. DEAN, M.A., M.B., Ch.B., M.R.C.P., Assistant Bacteriologist,
Lister Institute, London.

(Communicated by Dr. C. J. Martin, F.R.S. Received October 13,—Read
December 7, 1911.)

Of the various reactions which can be observed to take place between antigen and antibody, agglutination has usually been looked upon as relatively simple. It has been assumed that the clumping of bacteria or red cells is produced by the action of substances known as agglutinins. According to the well-known views of Ehrlich, an agglutinin is possessed of two groups, a cytophile or haptophore group which fixes on to the cell, and a group which has the property of producing agglutination. According to another view the cell or bacterium combines with its specific antibody, and the combination of cell and antibody is then clumped by the action of electrolytes. A broad distinction has, however, always been drawn between such phenomena as precipitation and agglutination, which appear to represent a comparatively simple reaction between antigen and antibody, and those more complex effects such as haemolysis and bacteriolysis in which another constituent of serum, the alexine or complement, is necessary to complete the specific action of the antibody.

Observations published by Muir and Browning (1906) suggested, however, that in some instances, at any rate, the mechanism of agglutination may be more complex.

It was found that fresh ox serum powerfully agglutinated a suspension of ox corpuscles in the presence of antiserum obtained from a rabbit which had been injected with ox corpuscles. The fresh serum could augment to a very marked degree the agglutinative properties of the homologous antiserum. This agglutination of red cells by immune body and complement took place rapidly at 37° C., and somewhat more slowly at room temperature: at 0° C. the agglutination was imperfect. In another experiment, however, complete agglutination was obtained at 0° C. by mixing the red corpuscles of a guinea-pig, the homologous antiserum derived from a rabbit, and fresh ox serum. The property possessed by ox serum of furthering agglutination was destroyed by heating the serum for an hour at 55° C.

In the same year Bordet and Gay (1906) gave an account of a series of very similar experiments. Bordet and Gay independently discovered the agglutinative property of ox serum for sensitised red cells. They found,

moreover, that, although this agglutinative property was lost by heating the ox serum at 56°C ., it could be restored by adding a little of the fresh serum (complement) of another animal. They concluded that there exists in ox serum a special substance which resists a temperature of 56°C ., and which may be preserved for many months in the heated serum. This substance, which was presumably of an albuminous or colloidal nature, showed no tendency to unite with normal corpuscles, but was precipitated on the corpuscles charged with the substance sensibilisatrice and alexine. They called this substance *Colloïde du bœuf*. This form of agglutination, which has received the name of conglutination, is attributed by Bordet to the action of three factors on the red corpuscles, namely: (1) the specific antiserum, (2) the ox colloid, (3) a fresh serum or alexine. The heat-resisting substance present in ox serum is called in subsequent papers conglutinin.

Bordet and Streng (1909) published a series of experiments dealing with the agglutinative properties of ox serum. They declared that the conglutinins were essentially different from the agglutinins. As a point of difference they stated that the conglutinins had no need to be fixed on the cells which they conglutinated. This statement appears to be at variance with the previously-quoted observation that the specific substance of the ox colloid is precipitated on corpuscles duly laden with substance sensibilisatrice and alexine.

Bordet and Streng also subjected ox serum after it had been heated to 56°C . to dialysis. They found that the fraction which remained in solution favoured hæmolysis, while the precipitate, if re-dissolved in normal saline, favoured agglutination.

In another communication Streng (1909) claims considerable success for the conglutination method in the identification and differentiation of bacteria. By the addition of ox serum and complement he obtained marked agglutination of bacteria with a dilution of homologous antiserum, which by itself was too weak to produce any trace of agglutination. Streng also stated that conglutinin could be separated from agglutinin by dialysis. The agglutinin, under these conditions, remained in solution, while the conglutinin was precipitated with the globulin fraction.

Barikine (1910) effected a similar separation of agglutinin and conglutinin by saturating ox serum with carbon dioxide. As in the dialysis experiment, the conglutinin was precipitated with the globulin, and the agglutinin remained in solution. Barikine also found that the flocculi of a specific precipitate, formed by the union of antigen and antibody, could be conglutinated by the addition of fresh serum (complement) and heated ox serum (conglutinin).

Bordet and Gengou (1911) published a paper dealing with a phenomenon which they have named co-agglutination, and which they have expressly stated is to be clearly distinguished from conglutination. They found that a mixture of antigen and antibody is able to produce a very marked agglutination of the red cells of a third animal, the guinea-pig. The conditions under which this co-agglutination occurs are carefully set forth by the authors. Guinea-pig blood was found to be the only sort of blood which gave satisfactory results. Defibrinated guinea-pig blood was, as a rule, employed, but equally satisfactory results could be obtained by the use of a suspension of washed corpuscles. Both the serum which contained the antigen and the serum which contained the antibody were heated to 56°C . before use. The co-agglutination was obtained with all the antigen-antibody systems used by the authors. As the co-agglutination was obtained by the use of heated sera and washed guinea-pig corpuscles, the participation of complement could be excluded. Co-agglutination only occurred if a considerable excess of antigen relative to antibody was present in the mixture. The proportions most favourable to co-agglutination were such that the antigen was present in such excess as to inhibit the formation of a precipitate. When the proportions were such that a marked precipitate formed, co-agglutination did not occur. The co-agglutination was not accompanied by any marked fixation of complement. It was necessary that the guinea-pig corpuscles should be present at the time when the antigen came into contact with the antibody. To produce this result the corpuscles were mixed with the antigen and the antibody was then added. Under the proper conditions the agglutination of the red corpuscles was not only very marked but very persistent, that is to say, the corpuscles could be shaken up an indefinite number of times and invariably re-agglutinated.

It would thus appear that the clumping or agglutination of red cells may take place under three different sets of conditions:—

(1) *Agglutination*.—By this is meant the well-known clumping of red cells by a specific antiserum or by a normal serum which possesses agglutinins for the red cells employed.

(2) *Conglutination*.—This is produced by the action of ox colloid (conglutinin) on cells which have been treated with homologous antiserum and with complement. In place of a serum prepared by the injection of an animal with red cells, a normal serum which contains a normal agglutinin for the red cells can, however, be employed.

(3) *Co-agglutination*.—An antigen and homologous antibody can under appropriate conditions agglutinate the red cells of another animal (preferably a guinea-pig).

As regards conglutination and agglutination it appears that the action of ox colloid and complement is to intensify the effect of an agglutinin present in a normal or an immune serum, such action being of the nature of complementing. It is necessary for conglutination that the cells should be sensitised. Muir and Browning, in fact, expressed the view that the ox serum acted as a complement to the immune serum.

The interaction of the various factors in agglutination and conglutination finds to some extent a parallel in phagocytosis, in which the action of a heated serum is intensified by the addition of complement.

General Object of the Experiments.

The experiments here recorded were undertaken as the result of a chance observation. A number of experiments had been made with the view of ascertaining the relative quantities of the two fractions of complement necessary for the production of hæmolysis. In such an experiment it is, of course, essential to put up control tubes which contain the various dilutions of the middle-piece and of the end-piece in order to make certain that neither fraction acting by itself can produce hæmolysis. It was noticed that the corpuscles in the middle-piece control tubes presented a remarkable appearance. Instead of settling down to form a small mass at the very bottom of the tube, the corpuscles were found to be arranged in a thin layer which coated the bottom end and lower part of the tube. The layer of corpuscles took the shape of the lower part of the tube and produced the appearance of a small cup. If the tube was sharply shaken it could be seen that the corpuscles had been agglutinated. The control tube which contained corpuscles and immune body without the middle-piece solution showed no agglutination. The agglutination had been produced by the joint action of the inactivated hæmolytic serum and middle-piece solution.

Method of Preparation of Experimental Material.

Preparation of Complement Fractions—

In the experiments which are to be described the complement fractions have been obtained by the carbon dioxide method of Liefmann (1909). Fresh guinea-pig serum is diluted with distilled water in the proportion of one part of serum to nine parts of distilled water. The mixture should be kept cold in an ice bath, and it is an advantage to prepare the mixture with ice-cold distilled water. The mixture is saturated with carbon dioxide and then allowed to stand for one hour in the cold room. The carbon dioxide produces a marked turbidity in the mixture, and at the end of the hour's

standing small flocculi are apparent. The precipitate is brought down with a centrifuge and resuspended in ice-cold distilled water. This process of washing the precipitate is repeated, and the precipitate is then dissolved in cold 0.85 per cent. sodium chloride solution. The experiments were performed during the summer months, and it was found that, unless care was taken to keep the original mixture and the suspension of precipitate as cold as possible, it was very difficult to redissolve the precipitate in the saline solution. The saline solution was used in such quantity that the resulting middle-piece solution corresponded to a 1 in 10 dilution of fresh guinea-pig serum. The supernatant fluid from which the precipitate had been removed was, as a rule, quite clear, but was generally filtered through filter paper to remove any trace of suspended particles. Sufficient sodium chloride was then added to make it equal to a 0.85 per cent. sodium chloride solution. The resulting solution which contained the end-piece fraction corresponded to a 1 in 10 dilution of the fresh guinea-pig serum.

Preparation of Other Materials—

The red corpuscles were obtained from the sheep. The blood was defibrinated with glass beads. The corpuscles were freed from serum by three washings with normal saline solution. The hæmolytic serum was obtained from a rabbit which had had three intravenous injections of washed sheep corpuscles. The serum was inactivated by heating for half an hour at 56° C.

The bacterial emulsions were prepared by emulsifying in normal saline a 24-hours agar culture of *B. typhosus*. The antityphoid serum was obtained from rabbits which had been immunised by intravenous injections of saline emulsions of *B. typhosus* (killed by heating for one hour at 60° C.).

The antityphoid serum was inactivated by heating for half an hour at 56° C.

Detailed Description of Experiments and Results.

Influence of the Middle-Piece on Agglutination—

An hæmolytic serum, produced by injecting a rabbit with the washed red corpuscles of a sheep, possesses, in addition to its hæmolytic properties, considerable power of producing agglutination. Agglutination of the red cells is, however, evident only if a rather large amount of antiserum be present in the mixture. In the case of the serum with which these experiments was performed it was necessary to employ the serum in a strength of at least 1 in 100 to obtain any marked degree of agglutination within a period of one hour. If the serum was employed in a dilution of 1 in 200 agglutination could not be detected. It may be mentioned that the hæmolytic titre of this serum was about 1 in 1000. If to a mixture of one volume of a

1 in 20 suspension of red cells, with one volume of a 1 in 200 dilution of heated antiserum, was added one volume of 1 in 10 middle-piece solution, an almost instantaneous and very marked agglutination of the red cells took place. On slanting the tube the red cells could be seen to be aggregated in large clumps. The clumps rapidly increased in size, and after 20 minutes to half an hour had settled to the bottom of the tube to form a single mass which somewhat resembled a soft clot. The supernatant fluid was left quite clear. The viscous mass which formed at the bottom of the tube could be disintegrated by vigorous shaking, but rapidly re-formed, and the process of shaking the clump apart and allowing it to re-form could be repeated indefinitely. One series of tubes was preserved for 48 hours without any change in the condition of the corpuscles. The appearances presented corresponded closely to the description recently given by Bordet and Gengou (1911) of the phenomenon which they called co-agglutination.

It must be plainly stated that the action of the solution of middle-piece is to accentuate the feebly agglutinative action of a small amount of specific antiserum. An agglutination quite as marked and apparently identical in nature could be produced by using a larger quantity of the antiserum without the addition of middle-piece. The action of middle-piece appeared

Table I.

	1 c.c. of dilution of hæmolytic serum, Rabbit & Sheep.	+ 1 c.c. middle-piece solution diluted.					+ 1 c.c. normal saline.
		1—10.	1—20.	1—40.	1—80.	1—160.	
1	1—10	++++	++++	++++	++++	++++	++++
2	1—20	++++	++++	++++	++++	++++	++++
3	1—40	++++	++++	++++	++++	++++	++++
4	1—80	++++	++++	++++	++	++	++
5	1—160	++++	++++	+++	++	+	+
6	1—320	++++	++++	++	+	0	0
7	1—640	+++	++	+	0	0	0
8	1—1280	+++	+	0	0	0	0
9	1 c.c. normal saline	0	0	0	0	0	0

Each tube contained a volume of 3 c.c. made up of 1 c.c. of a 1 in 20 suspension of washed red cells of the sheep, 1 c.c. of a dilution of heated hæmolytic serum (rabbit and sheep), and 1 c.c. of the diluted middle-piece solution. The tubes numbered 9, in each row, contained no immune serum and the bulk was made up to 3 c.c. by the addition of 1 c.c. of normal saline solution. In these tubes no agglutination occurred, the middle-piece solution by itself being unable to agglutinate the red cells. The tubes in the last column contained 1 c.c. of the suspension of red cells, 1 c.c. of a dilution of the hæmolytic serum and 1 c.c. of normal saline solution. The agglutinative power of the immune body acting by itself is shown in this column. In the remaining columns is shown the effect of the combined action of the immune body and the middle-piece solution.

to be to enormously increase the action of a dilution of antiserum, which if acting by itself would have produced a hardly perceptible degree of agglutination.

The solution of middle-piece was shown by repeated experiments to have no agglutinative action on the red corpuscles in the absence of the antiserum. It is evident from Table I that a very marked degree of agglutination may be produced by the interaction of three components—the red cells, the heated homologous antiserum, and the solution of middle-piece.

Remarks on Table I.

From a consideration of Table I it appears probable that for the agglutination of the red cells two substances are necessary, the one being the specific antibody to the red cells and the other a non-specific substance. Both of these substances are thermostable, and are present in inactivated hemolytic serum. The larger quantities of the antiserum contained, in addition to the specific antibody, a sufficient quantity of the non-specific substance to produce agglutination of the red cells. If a smaller quantity of the antiserum was used, the amount of non-specific substance was insufficient. In such cases the necessary substance could be supplied by the addition of the solution of middle-piece. An effect of this type is illustrated in Table II.

In this experiment an amount of antiserum was employed which, acting by itself, was unable to agglutinate the quantity of red cells present. Such a quantity of antiserum was found to be 1 c.c. of a 1 in 200 dilution.

Table II.

	1 c.c. of dilutions of middle-piece solution.	1 c.c. of 1—200 dilution of antiserum.	1 c.c. of 1—20 suspension washed red cells.	
1	1—10	1—200	1—20	++++
2	1—20	"	"	++++
3	1—40	"	"	++++
4	1—80	"	"	++++
5	1—160	"	"	+++
6	1—10	1 c.c. normal saline	"	0
7	1 c.c. normal saline	1 c.c. 1—200	"	0

Neither the antiserum alone, in a 1 in 200 dilution, nor the solution of middle-piece was able by itself to agglutinate the red cells. The two factors in combination produced a very marked degree of agglutination.

Experiment to Show that the Agglutinative Properties of the Middle-Piece Solution are Thermostable.

The explanation offered in the preceding paragraph assumes that the agglutinating power of an immune serum depends on the presence of two thermostable substances, namely, the specific antibody and a non-specific substance. It is suggested that a deficiency of the non-specific substance in a greatly diluted antiserum may be made good by the addition of middle-piece solution.

Before this explanation can be accepted it is necessary to show that this substance or property of the solution of middle-piece is thermostable, that is to say, capable of resisting a temperature of 56° C. for half an hour. The result of an experiment intended to settle this point is given in Table III.

Table III.

	Dilutions of the middle-piece solution.	Fresh solution.	Middle-piece solution previously heated at 56° C.			
			½ hour.	1 hour.	2 hours.	4 hours.
1	1—10	++++	++++	++++	++++	++++
2	1—20	++++	++++	++++	++++	++++
3	1—40	++++	++++	+++	+++	+++
4	1—80	++++	++++	+++	++	++
5	1—160	++	++	++	+	+

Quantities of 5 c.c. of the middle-piece solution were heated at 56° C. for ½, 1, 2, and 4 hours. Parallel dilutions of each sample were then made, and of the original unheated solution. To each tube, which contained 1 c.c. of diluted middle-piece solution, was added 1 c.c. of sheep cells 1 in 20, and 1 c.c. of a 1 in 200 dilution of hæmolytic serum. Control tubes were put up which showed that neither a 1 in 200 dilution of hæmolytic serum nor a 1 in 10 dilution of middle-piece was capable, when acting by itself, of agglutinating the red cells.

Remarks on Tables III and IV.

From this and similar experiments, it was determined that the capacity of the solution of middle-piece to aid in agglutination was only very gradually destroyed at a temperature of 56° C.; heating for half-an-hour had a very slight, or no effect at all, in reducing its activity.

This property of aiding in agglutination can be classed as one of the relatively thermostable properties of serum. It is equally evident that this property of the solution of middle-piece has no connection with its hæmolytic property, for the latter is rapidly lost by subjecting such a solution to a temperature of 56° C. The middle-piece fraction of the hæmolytic complement is definitely thermolabile. On the other hand, whole guinea-pig

serum, which had been inactivated in the ordinary way by heating it for half-an-hour at 56° C., was found to possess to a marked degree the property of increasing agglutination. As far as agglutination was concerned, the heated whole guinea-pig serum appeared to possess the same properties as the saline solution of middle-piece.

Table IV.—Comparison of Heated Whole Serum with the Middle-Piece Fraction.

The fresh guinea-pig serum was previously heated for half an hour at a temperature of 56° C.

Dilutions.		Heated whole serum.	Middle-piece solution.
1	1—10	+	+
2	1—20	+	+
3	1—40	+	+
4	1—80	+	+
5	1—160	+	+

The two sets of dilutions were comparable, that is to say, the 1 in 10 dilution of middle-piece solution corresponded to the amount of middle-piece in a 1 in 10 dilution of whole serum.

To every tube was added a suspension of red cells and the diluted immune serum. It was shown by appropriate controls that neither this dilution of immune serum, 1 in 200, nor the heated whole serum, nor the middle-piece solution, in the dilutions employed, had the power of agglutinating the red cells.

Influence of the End-Piece.

Only a few experiments were carried out with a view to ascertaining the influence of the end-piece fraction of the complement. In one case the solution of end-piece had no influence on agglutination, acting either alone or in conjunction with the middle-piece solution. Another end-piece solution had a slight influence in a dilution of 1 in 10 in increasing the agglutination of the red cells by an antiserum. When further diluted it had no action. The same solution of end-piece somewhat increased the agglutination produced by the interaction of antibody and middle-piece.

It should be added that neither solution of end-piece had in the dilutions employed the slightest agglutinative action on the red cells in the absence of the specific immune body. The discrepancy between the two solutions probably depended on slight differences in the method of separation.

In any case, the substance which aids in agglutination appears to be contained chiefly in the fraction of the globulin which is precipitated by carbon dioxide. The agglutinating substance is present in whole normal serum, but its agglutinative property can be conveniently studied in a middle-piece solution, since the progress of agglutination is not interrupted

by the lysis of the red cells. For this reason a middle-piece solution prepared from fresh guinea-pig serum was used in the majority of the experiments.

Addition of Middle-Piece to Sensitised Cells.

The action of the solution of middle-piece can be demonstrated in a very striking manner by adding middle-piece solution to corpuscles previously sensitised with the homologous antiserum.

One cubic centimetre of a 1 in 20 suspension of sheep red cells was added to 1 c.c. of a 1 in 200 dilution of antiserum. The mixture was allowed to stand for half-an-hour at room temperature. At the end of this time there was no evidence of agglutination. There was then added 1 c.c. of a 1 in 10 solution of middle-piece. The red cells immediately ran together into large clumps and rapidly settled to the bottom of the tube. This experiment showed that the middle-piece solution could exert its action on already sensitised red cells, and that it was not essential that the middle-piece should be present from the time of the first admixture of antigen and antibody. The sensitised red cells can, in fact, if freed by repeated washings from every trace of uncombined antibody, be still agglutinated by the addition of middle-piece solution. Previously sensitised red cells are, in fact, agglutinated with great rapidity, for time is not taken up by the union of the red cells with the antibody.

Influence of Temperature on the Reaction.

No strictly comparative experiments have as yet been undertaken with a view to ascertaining the influence of temperature on the agglutination of red cells by immune body and middle-piece solution. The majority of experiments were carried out at room temperature, but the agglutination was somewhat accelerated by placing the tubes in an incubator at 37° C. On the other hand, it was ascertained that a very marked degree of agglutination was reached when the tubes were placed in the cold room at a temperature of a few degrees above 0° C. The middle-piece solution is certainly able to agglutinate sensitised corpuscles in the cold, and the delay in agglutination is sufficiently explained by the longer time required at a low temperature for the union of the red cells with antibody.

Agglutination of Bacteria.

A considerable number of experiments were made with the object of reproducing with bacterial emulsions the results obtained by the use of blood corpuscles. In these experiments an inactivated antityphoid serum derived from a rabbit and an emulsion in normal saline of a 24-hours' agar

culture of *B. typhosus* were used. Such an experiment is represented in Table V.

Table V.

	0.5 c.c. of inactivated diluted antityphoid serum.	A. + 1 c.c. normal saline.	B. + 0.5 c.c. middle- piece 1—10, and 0.5 c.c. normal saline.	C. + 0.5 c.c. middle- piece 1—10, and 0.5 c.c. end-piece 1—10.
1	1 in 4000	++	+++	+++
2	1 in 5000	+	+++	+++
3	1 in 6000	0	++	++
4	1 in 7000	0	++	++
5	1 in 8000	0	+	+
6	1 in 9000	0	0	0
7	Controls 0.5 c.c. normal saline	0	0	0

Every tube contained 2 c.c. In the control tubes the bulk was made up to 2 c.c. with normal saline. Tube 7 contained in column A 0.5 c.c. of bacillary emulsion and 1.5 c.c. of saline; in row B emulsion and middle-piece; in row C emulsion and middle-piece and end-piece. In these controls no agglutination was observed. In row A the agglutinative power of the immune serum is recorded. No agglutination was observed below tube 2. It will be seen that this tube contained 0.5 c.c. of emulsion of bacteria, 0.5 c.c. of a 1 in 5000 dilution of antityphoid serum, and 1 c.c. of normal saline solution. The ultimate dilution of the immune serum was in consequence 1 in 20,000. Column B shows the agglutination produced by immune serum in the same dilutions plus 0.5 c.c. of a 1 in 10 dilution of middle-piece in every tube. All the tubes were incubated for 4 hours at 37° C.

Remarks on Table V.

It will be seen that the agglutinative power of the immune serum is increased by the addition of middle-piece. The result of the addition of end-piece to middle-piece and immune serum is shown in Column C. No further increase in agglutination was observed. Numerous experiments were made which gave results consistent with those shown in Table V.

To demonstrate the effect of middle-piece solution it is necessary to dilute the immune serum to such an extent that its agglutinative power is just beginning to disappear. The action of such diluted antiserum can be increased by the addition of middle-piece solution. Further, the agglutinative power of an antiserum which has been diluted beyond the point at which the agglutination can be appreciated, can be restored by the addition of the middle-piece solution.

These experiments show that there exists in normal guinea-pig serum some substance which increases the agglutinative action of antiserum. This substance is thermostable, and probably has no connection with complement. It can be separated from serum with the fraction of the globulins which are

precipitated by diluting a serum with distilled water and saturating the mixture with carbonic acid. In these characteristics it conforms to the description of conglutinin which is given by Bordet and Streng. The substance may be neither more nor less than serum globulin or some fraction of globulin which is easily precipitated.

The experiments suggest that an agglutinating serum contains a specific antibody and a non-specific substance, both of which are necessary to produce agglutination. When such a serum is greatly diluted the dilution may contain sufficient of the specific antibody but not sufficient of the non-specific anti-substance. In such a case the non-specific substance can be supplied by adding middle-piece solution prepared from normal serum, and agglutination is produced.

Experiments to Determine the Way in which the Middle-Piece Solution aids in Agglutination.

The inter-relation of the factors concerned in agglutination is to some extent illustrated by the following experiment. Ten cubic centimetres of a 1 in 20 suspension of washed sheep red corpuscles were mixed with 20 c.c. of a 1 in 200 dilution of a heated haemolytic antiserum. The mixture was allowed to remain in the cold room for one hour and was then centrifugalised. The corpuscles were then thoroughly washed and again centrifugalised. The sensitised corpuscles were then mixed with 10 c.c. of middle-piece solution. In another tube an equal quantity of unsensitised sheep corpuscles was added to 10 c.c. of middle-piece solution. Both tubes were placed in the cold room. At the end of this time very marked agglutination had taken place in the tube containing the sensitised corpuscles. The contents of both tubes were centrifugalised. The deposit of agglutinated cells was shaken up in normal saline and again centrifugalised. After two washings the deposit was thoroughly shaken up and suspended in normal saline. Within 15 minutes the cells had re-agglutinated, and after half an hour had fallen in a compact mass to the bottom of the tube.

The supernatant fluid from the tube which contained the sensitised red cells was added to a further quantity of sensitised red cells. After the lapse of several hours a very slight degree of agglutination could be detected. It is evident that sensitised red cells can, in the process of agglutination, remove from the solution of middle-piece the substance which produces the agglutination. The supernatant fluid from the tube which had contained the unsensitised red cells contained the agglutinative substance in a state of unimpaired activity. Some substance had been taken out of solution by the sensitised but not by the unsensitised cells.

The above experiment shows that sensitised cells remove from a middle-piece solution the substance which causes their agglutination. To demonstrate the manner in which this substance was removed it was decided to employ a solution of the constituents of the corpuscles in distilled water. One cubic centimetre of thoroughly washed sheep corpuscles was laked with 9 c.c. of distilled water. The solution of corpuscles was made up to the usual saline content by the addition of 10 c.c. of 1.7 per cent. sodium chloride solution. After filtering many times through filter paper a perfectly clear solution was obtained, representing a 1 in 20 solution of red corpuscles in normal saline.

The following mixtures were then prepared :—

Tube.	Solution of laked corpuscles.	Antiserum, rabbit <i>v.</i> sheep cells, 1 in 200.	Normal saline solution.	Solution of middle-piece 1 in 10.	Normal saline solution.
	c.c.	c.c.	c.c.	c.c.	c.c.
1	5	5	—	10	—
2	5	—	5	10	—
3	5	5	—	—	10
4	—	5	5	10	—

In tube 1 5 c.c. of the solution of corpuscles was added to 5 c.c. of the diluted antiserum. In the control tubes 2, 3, and 4 the ingredients indicated in the first three columns were mixed. The volume in each tube was then 10 c.c. All the tubes were allowed to remain for one hour at room temperature. They were then examined and the contents were found to be absolutely clear. To tubes 1, 2, and 4 were then added 10 c.c. of the 1 in 10 middle-piece solution; to tube 3 was added 10 c.c. of normal saline solution. All four tubes were then incubated for four hours at 37° C., and then placed for 12 hours in a cool chamber at about 8° C. Tube 1 was then found to contain a small but definite white flocculent deposit. The three control tubes remained absolutely clear.

This experiment with unimportant variations in detail was several times repeated. In every case the tube which contained antigen, antibody, and middle-piece solution contained a precipitate. The control tubes contained no precipitate.

Results obtained by adding Middle-Piece Solution to a Mixture of a Normal Serum with its Homologous Antiserum.

It has been shown that sensitised red corpuscles are agglutinated by middle-piece and that in the process of agglutination the substance which produces

the agglutination is removed from solution. It has also been shown that a precipitate is formed in a mixture of laked corpuscles, antibody, and middle-piece. This suggests that the substance present in the middle-piece solution is actually precipitated on the sensitised corpuscles and that such precipitation is a part of the mechanism of agglutination.

It was decided to examine the effect of adding middle-piece solution to a

Table VI.

3 c.c. of horse serum (antigen) diluted.		+ 3 c.c. antiserum, rabbit <i>v.</i> horse, diluted 1 in 10.		+ 3 c.c. antiserum, rabbit <i>v.</i> horse, diluted 1 in 100.		+ 3 c.c. normal saline solution.	
		(A) + 3 c.c. normal saline.	(a) + 3 c.c. middle-piece 1 in 10.	(B) + 3 c.c. normal saline.	(b) + 3 c.c. middle-piece 1 in 10.	(C) + 3 c.c. normal saline.	(c) + 3 c.c. middle-piece 1 in 10.
1	1 in 5	Large precipitate	Large precipitate	Clear	Clear	Clear	Clear
2	1 in 10	"	"	Very slight opalescence	Opalescence	"	"
3	1 in 20	"	"	"	Marked turbidity	"	"
4	1 in 40	"	"	Clear	"	"	"
5	1 in 80	"	"	"	Opalescence	"	"
6	1 in 160	"	"	"	Slight opalescence	"	"
7	1 in 320	Precipitate	Precipitate	"	Clear	"	"
8	1 in 640	"	"	"	"	"	"
9	1 in 1280	Turbidity	Turbidity	"	"	"	"
10	Controls. 3 c.c. saline solution	Clear	Clear	Clear	Clear	Clear	Clear

All the tubes contained 9 c.c. The tubes numbered 1 to 9 in each column contained various dilutions of normal horse serum (antigen). To the tubes in column A were added 3 c.c. of normal saline solution and 3 c.c. of antiserum in a dilution of 1 in 10. To the tubes in row *a* were added 3 c.c. of a middle-piece solution (1 in 10) and 3 c.c. of antiserum 1 in 10. A bulky precipitate was formed in the tubes of column A and of column *a*. There was no difference in the appearance of the tubes in column A and in the tubes in column *a*. To the tubes in column B were added 3 c.c. of normal saline and 3 c.c. of antiserum diluted 1 in 100. To the tubes in column *b* were added 3 c.c. of middle-piece solution 1 in 10 and 3 c.c. of antiserum diluted 1 in 100. This dilution of antiserum produced a rather doubtful opalescence in tubes 2 and 3 of column B. The remaining tubes of column B were clear. A very distinct difference existed between the tubes in column B and the tubes in column *b*. In the tubes in column *b* opalescence or turbidity was distinctly visible in tubes 2 to 6. The tubes in column C contained horse serum only. The tubes in column *c* contained horse serum and middle-piece. No precipitate was produced by this mixture. The tubes marked 10 in the various columns show that neither antiserum alone nor antiserum and middle-piece solution produced a precipitate.

It will be noticed that the tubes 1 in columns B and *b* showed no opalescence. This inhibitory effect is due to relative excess of antigen.

The tubes were incubated for 4 hours at 37° C., and then allowed to stand overnight in the cold room.

mixture of normal horse serum and the serum of a rabbit which had been injected with horse serum. Both the horse serum and the antiserum were heated for half an hour at 56° C. before use. Since such a mixture of serum and antiserum produces a bulky precipitate it was found necessary to dilute the antiserum to such an extent that only a slight trace of opalescence was produced when it was mixed with the antigen. The antiserum used was found to give a hardly perceptible opalescence in a dilution of 1 in 100. If less diluted antiserum was employed the precipitate formed was so large as to make it impossible to determine if the middle-piece solution took any part in the reaction. The result of such an experiment is shown in Table VI.

Remarks on Table VI.

From a consideration of Table VI it appears that a mixture of a normal serum with its homologous antiserum is able to effect the precipitation of some substance present in the middle-piece solution. This effect can only be demonstrated by using a greatly diluted antiserum. As in the agglutination experiments the effect of adding middle-piece solution was only demonstrable if it was added to a small quantity of antiserum. It also appeared that for the effective precipitation of middle-piece it was necessary that there should be present a relative excess of antigen. This is of interest since it is precisely under these conditions that Bordet was able to produce the phenomenon which he called co-agglutination.

On the Properties of the Globulin Solution prepared from Sheep Serum.

The capacity of the middle-piece or globulin solution obtained from guinea-pig serum does not appear to be a peculiarity of the serum of the guinea-pig. Fresh sheep serum was treated with CO_2 in a similar fashion and the resulting solution of the globulin precipitate had the power of increasing agglutination in a suitable mixture of sheep corpuscles and hamolytic antiserum. The result of adding a solution of sheep globulin to a mixture of horse serum with rabbit *v.* horse serum is shown in Table VII.

The following mixtures were prepared:—

Table VII.

	Normal horse serum diluted 1 in 10.	Rabbit <i>v.</i> horse serum diluted 1 in 50.	Sheep middle- piece solution 1 in 10.	Normal saline solution.
	c.c.	c.c.	c.c.	c.c.
1	5	5	10	—
2	5	—	10	5
3	—	5	10	5
4	5	5	—	10

All four tubes were incubated for six hours at 37° C. and then allowed to stand for 12 hours in the cold room. A distinct turbidity formed in tube 1. The contents of the remaining three tubes remained perfectly clear.

The middle-piece or globulin solution obtained from sheep serum was shown to have the same properties as the middle-piece solution obtained from guinea-pig serum. It is proposed to supplement these experiments by examining the properties of the globulin solutions of a variety of animals.

Discussion of Results.

An agglutinating serum contains two factors, both of which are necessary to agglutination. The one is the specific antibody, the other a precipitable substance, probably of the nature of a globulin. By the interaction of antigen and antibody the molecules of the precipitable substance are aggregated on the surface of the blood corpuscle or bacterium which is to be agglutinated.

The amount of specific antibody necessary to produce agglutination is probably minute, and, by diluting an antiserum, a dilution can be obtained which contains sufficient antibody but not sufficient of the precipitable substance. By adding to such a dilution of the antiserum a solution of the precipitable substance, derived from normal guinea-pig serum, agglutination can be effected. The amount of precipitable substance necessary to produce the agglutination of sheep corpuscles appears to be considerably larger than the amount required to agglutinate typhoid bacilli. The precipitable substance is thermostable, it is present in heated normal serum, and it can be precipitated from normal serum with a fraction of the serum globulin. It can also be precipitated from a solution in normal saline by a suitable combination of an antigen with its antibody. This precipitate is small and does not become visible until the experiment has been incubated for several hours.

It is probable that agglutination is effected during the earlier stages of the aggregation of the molecules of the precipitable substance, that is to say, before the process has advanced to the stage when a turbidity is visible. The precipitable substance is probably identical with "conglutinin." There is, however, this difference between the results obtained in these experiments and the conglutination effects of Bordet and his collaborators.

In Bordet's experiments, conglutination was obtained by the interaction of four factors, namely, the red cells, the heated antiserum, heated ox serum (conglutinin), and complement. In the experiments described in this paper, agglutination was effected by the interaction of three factors—the red cells,

the heated antiserum, and the substance present in the middle-piece fraction of guinea-pig complement.

Sufficient experiments have not been performed to justify a definite statement as to the relation of the phenomena of conglutination and agglutination. Nevertheless, it seems possible that agglutination and conglutination are essentially the same process. This process is the aggregation or precipitation of a precipitable substance by the interaction of antigen and antibody. In the case of agglutination this substance is a constituent of the agglutinating serum. In the case of conglutination a further supply of this substance is supplied from another source (ox serum).

The phenomenon described under the name of co-agglutination is of great interest in that the antigen is not a constituent of the agglutinated cell, but is derived from some different source. In such an experiment the interaction of antigen with antibody produces such a change in the physical conditions of the mixture that the suspended corpuscles, which may be supposed to have no affinity for the antigen or antibody, are spontaneously agglutinated. The result suggests the possibility that in an ordinary agglutination experiment the corpuscles may be agglutinated as the result of a reaction between antibody and antigen, which has diffused out of the corpuscle into the surrounding fluid. If such a view be correct, it follows that the phenomena described as agglutination, conglutination, and co-agglutination are essentially the same.

Apart from the question of agglutination, the results recorded may possibly be found to have some bearing on other serum reactions. The influence of the middle-piece and end-piece fractions of the complement in phagocytosis has been investigated by Dr. Ledingham in conjunction with the author, and the results of these experiments are shortly to be published.

With regard to the formation of precipitates, the experiments suggest that a suitable mixture of serum and antiserum is capable of precipitating a non-specific substance derived from the serum of a third animal. It seems, indeed, probable that the reason why an antiserum, if diluted, loses its power of producing a precipitate is not because the dilution contains too little antibody, but because there is not sufficient precipitable substance present to produce a precipitate.

It is sometimes held that, because a mixture of antigen with a dilution of antiserum can be prepared which shows no precipitate and nevertheless efficiently binds complement, the complement-binding antibody must be different from the precipitate-forming antibody. Now it has been shown in Table VI that a mixture of certain proportions of horse serum with its homologous antiserum may remain quite clear, while on the addition of the

globulin solution of guinea-pig serum a turbidity appears. Now this globulin solution contains the middle-piece fraction of guinea-pig complement, the fraction which is known to disappear in a complement-fixation experiment. It is proposed, therefore, to make these questions the subject of further investigation.

Summary.

(1) Sheep corpuscles are, as is well known, agglutinated by an homologous antiserum. If, to a mixture of corpuscles with antiserum so dilute that no agglutination is visible, there be added a solution of globulin obtained from normal guinea-pig serum, the corpuscles are markedly agglutinated. By the use of suitable controls it can be demonstrated that neither the globulin solution nor the dilution of antiserum employed are of themselves capable of agglutinating the corpuscles.

(2) The substance present in the globulin solution which aids agglutination is relatively thermostable, and its presence can be demonstrated in whole heated guinea-pig serum.

(3) Corpuscles which have been sensitised and washed to remove free antibody can be agglutinated by the globulin solution. If, after agglutination has taken place, the corpuscles be removed with a centrifuge, the supernatant fluid can be shown to have lost its agglutinating property.

(4) The agglutinating power of an extremely dilute antityphoid serum can be increased by the addition of the globulin solution. By the addition of globulin solution to a mixture of emulsion of *B. typhosus* with a dilution of antiserum which is too weak by itself to agglutinate the bacilli, distinct agglutination can be obtained.

(5) The formation of a specific precipitate by the interaction of a serum with its homologous antiserum depends, as is well known, on the presence in the mixture of a relatively large amount of the antiserum. If, to a mixture of serum with antiserum so diluted that it is no longer able to produce a precipitate, is added the globulin solution, a definite turbidity is produced.

(6) It seems probable that an agglutinating serum (antiserum) contains two factors, both of which are necessary to produce agglutination. The one of these is the specific antibody, the other is a non-specific substance which is possibly serum globulin. The interaction of antigen with antibody produces an aggregation of the molecules of the non-specific substance which may ultimately result in the formation of a definite turbidity. This process of aggregation of the particles of the non-specific substance is an essential part of the process of agglutination. It is possible to make a dilution of an antiserum which contains sufficient of the specific anti-substance but not sufficient

of the non-specific substance. The deficiency in non-specific substance can be made up by the addition of a globulin solution obtained from normal serum.

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The Relation between the Fixation of Complement and the Formation of a Precipitate.

By H. R. DEAN.¹

ONE of the most interesting problems connected with immunity is the explanation of the process by which complement is absorbed by the mixture of an antigen with its homologous antibody.

One theory explains the phenomenon by assuming the existence of an amboceptor or interbody with two affinities, the one of which unites with the cell to be dissolved, while the other unites with the complement. According to the other view the complement is taken up by the complex formed by the union of cell and antibody, the antibody being supposed to act somewhat after the manner of a mordant.

The fixation of complement experiments of Bordet and Gengou have naturally received two different explanations. According to the one view the complement is fixed by the precipitate which is formed by the union of antigen and antibody. According to the other view, precipitation and complement fixation are two entirely separate and independent phenomena. The fixation of complement is attributed to the action of an amboceptor, and some workers have gone so far as to state that a special variety—"a complement-fixing amboceptor"—exists. This amboceptor, which they call a *Bordet amboceptor*, is considered to be distinct from the amboceptor concerned in bacteriolysis.

The controversy which arose between the supporters of these two explanations resulted in the publication of a large number of papers. Only the more important of these communications are here summarized.

In 1901, Bordet and Gengou showed that alexin was fixed by the mixture of a bacillary emulsion with its homologous antiserum. In the same year, Widal and Le Sourd (1901) confirmed this result, and succeeded in demonstrating that the serum of typhoid fever patients would bind complement in the presence of an emulsion of *Bacillus*

¹ From the Bacteriological Laboratory, Lister Institute. The first part of this paper was read at the meeting held on March 21.

typhosus. In 1902, Gengou extended the scope of the reaction by showing that the serum of animals which had been injected with a foreign proteid had acquired the property of fixing complement if mixed with that proteid. Despite the importance of this discovery, however, the next two years were not productive of any substantial addition to the literature of the subject. Interest in the question appears to have been revived by Moreschi (1905), who pointed out that the results which had been attributed to the action of anti-complements were in reality due to the fixation of complement by a mixture of antiserum with its homologous proteid. In the same year (1905), Neisser and Sachs applied the principles laid down by Moreschi to the production of a practical method for the identification of the blood of different animals. In subsequent papers Moreschi (1906) and Moreschi and Pfeiffer (1906) advanced the view that the complement was taken up by the precipitate. Moreschi concluded that precipitin and precipitinogen unite in variable proportions, and form a series of precipitates, which possess a more or less strong anti-complementary action. About the same time, and independently of Moreschi, the same explanation was put forward by Gay (1905), who came to the conclusion that the precipitate fixed the complement. In support of a criticism of the views of Neisser and Wechsberg (1901), it was shown by Gay (1905) that the amount of interference with hæmolysis was in proportion to the amount of precipitate formed, and this to the amount of antiserum present. Klein (1905) found the fixation of complement closely associated with the formation of a precipitate, and took up essentially the same position as Moreschi and Gay.

On the other hand, Neisser and Sachs (1906) found that the amount of precipitate and the capacity for binding complement stood in no direct proportions. They were able to obtain marked fixation of complement when no precipitate could be recognized. They attributed the fixation of complement to a union of the complement with the complementophile group of an amboceptor, in strict accordance with the theory of Ehrlich. The question has been critically reviewed by Muir and Martin (1906), who added the results of their experiments on the subject. Muir and Martin found that the fixation of complement was as a rule closely associated with the formation of a precipitate, but were not convinced that precipitation formed a complete explanation of complement fixation. They cited as an instance a rabbit v. guinea-pig serum which produced, when mixed with its homologous antigen, a faint cloudiness, but no definite precipitate. The capacity for fixing

complement, however, was possessed by this serum to a very high degree. In other experiments they found that, if the antigen was greatly diluted, no precipitate was formed, but the mixture was able to bind complement efficiently. They also observed during the process of immunizing a rabbit with human serum that the complement-binding properties of the antiserum could be demonstrated at an earlier date than the precipitating properties. Similar results were obtained by Altmann (1910), who immunized a series of rabbits with *Bacillus paratyphosus* B. In many cases it was found that the serum of these animals developed complement-binding properties before they could be shown to have developed agglutinating properties. In the case of one rabbit only, the agglutinating property was the first to appear. After a second injection it was found that the complement-fixing property of the serum was lost during the two days immediately following, while the agglutinating property during the same period showed only a reduction. When rabbits were injected with *Bacillus typhosus*, it was found that the development of the two properties ran a fairly parallel course, though there was some tendency for the agglutinating property to appear earlier than the complement-fixing property. When *Bacillus coli* was used for immunization, the authors found that the power to fix complement appeared earlier in the case of nine rabbits, but in the case of two rabbits marked agglutinating power developed, but little or no power to fix complement. Altmann concluded that the discrepancy was due to differences in the individual strains of bacteria. He considered that certain strains of *Bacillus coli* tended to induce the production of agglutinins, while others tended to produce complement-binding antibodies. Altmann regards these experiments as evidence that the agglutinins are distinct from the antibodies which are responsible for complement fixation.

Wassermann and Bruck (1905), who also consider that complement fixation is due to amboceptor action, adopted a different method with the object of supporting their view. A bacterial extract was prepared, which, when mixed with its homologous antiserum, produced a precipitate, and also a fixation of complement. This extract was set aside, and, after the lapse of some time, was found to have lost its power of producing a precipitate without any loss of its complement-binding properties. This experiment is quoted by Sachs and Altmann (1909) as conclusive proof of the separate identity of precipitins and complement-binding amboceptors.

Evidence of a similar nature was obtained by Friedberger (1906).

This observer heated an antiserum, obtained by injecting a rabbit with sheep serum, for one hour at a temperature of 67° C. This heated serum was found to have lost the property of forming a precipitate, while the property of fixing complement had been retained.

By injecting a rabbit with human serum, Friedberger obtained an antiserum so powerful that complement fixation could be obtained by the use of extraordinarily small quantities of the antigen (human serum 1 in 1,000,000,000). When these extraordinarily small amounts of antigen were used no trace of a precipitate could be detected, complement was nevertheless efficiently fixed.

Liefmann (1906) published a series of experiments, the general object of which was to obtain conditions under which complement fixation occurred without the formation of a precipitate. In the majority of his experiments he found that the two reactions were closely associated. He found, however, that if a serum (antigen) was heated, it lost its property of forming a precipitate when mixed with the homologous antiserum. Nevertheless, a mixture of the heated serum with the antiserum produced a marked fixation of complement. Liefmann concluded that the data at his disposal were insufficient to permit of a definite opinion being formed.

In a subsequent paper, Moreschi (1906) appears to have obtained results which are at variance with his earlier experiments. He prepared antisera by injecting hens and ducks. The antisera so obtained gave good precipitates, but bound no complement.

Other instances of precipitation without complement fixation have been recorded by Sobernheim (1906), who experimented with anti-tuberculous sera, obtained by the injection of tubercle bacilli and tuberculin.

For a complete review of the subject, as well as for further references to its literature, the reader is referred to the articles on complement fixation by Meier (1909) and Sachs and Altmann (1909).

The above summary shows that the opinion of the majority, and perhaps the bulk of the evidence, is in favour of the view that the complement-binding bodies are distinct from the precipitins. The evidence for the separate identity of precipitins and complement-binding amboceptors falls under four headings:—

- (1) Complement fixation can be demonstrated in mixtures which contain so small a quantity of antigen that no precipitate can be detected.

- (2) Certain antisera have been observed to give a precipitate, with little or no complement fixation.

Other antisera have good complement-binding properties, but form little or no precipitate.

(3) If either the antiserum or the normal serum (antigen) is heated, the property of forming a precipitate is lost, but the property of fixing complement remains. If a bacterial extract is kept for a time it loses its property of forming a precipitate with the homologous antiserum, but retains the property of fixing complement.

(4) During the process of immunization the complement-fixing property has been found to appear before the property of forming a precipitate.

OBJECT OF THE EXPERIMENTS.

The general object of these experiments has been to determine whether any relationship exists between the amount of precipitate formed and the amount of complement fixed. Before describing the experiments it seems advisable to refer briefly to the experiments of Chapman and Welsh. The papers which have been quoted were written at a time when it was generally assumed that the antiserum, the so-called precipitin, precipitated the antigen, the so-called precipitinogen. It was, in fact, generally held that the precipitate was derived from the antigen. Chapman and Welsh (1906-11) have, however, shown by numerous and exact experiments that the precipitate is almost, if not entirely, derived from the antiserum. It is the antiserum, in other words, which is precipitated by the antigen. It follows, then, that the amount of the precipitate is proportional to the amount of antiserum present. If, then, the formation of a precipitate is the cause of the fixation of complement, it becomes necessary to show that the amount of complement fixed is proportional to the amount of antiserum present. As already stated, Gay arrived at this conclusion in his explanation of the deviation effects produced by excess of antiserum in hæmolysis. It is not, however, an easy matter to show that this relationship exists, for, as will be seen later, the amount of the precipitate is not the only factor which determines the amount of complement fixed.

It is necessary to refer to one other point in connexion with precipitation. Chapman and Welsh found that a considerable time (up to ninety-six hours) was necessary for the complete precipitation of a given quantity of antiserum. In a complement fixation experiment it is obviously necessary to allow only a short time (about one hour) for the reaction. In the experiments to be described it was desired to compare the amount of complement fixed with the amount of

precipitate formed. To do so it became necessary to estimate the amount of precipitate formed during the time allowed for the fixation of complement. The figures given in the tables represent the precipitate formed in a given time, and do not necessarily represent the entire precipitable content of the amount of antiserum employed.

THE METHOD OF THE PREPARATION OF ANTISERA AND OTHER MATERIAL USED IN THE EXPERIMENTS.

Very little need be said as to the preparation of the antisera, which were all obtained from rabbits which had been immunized, either by the intravenous or the intraperitoneal method. Intravenous injection was used in nearly every case, and in most cases some four to six injections were given at intervals of four days. The sera were inactivated before use by heating for half an hour at 56° C. The antigens employed were: egg-white, horse serum, goat serum, human serum, monkey serum, and emulsions in normal saline solution of a twenty-four-hours' agar culture of *Bacillus typhosus*. The bacterial extracts used in some of the experiments were prepared by a method described in the *Proceedings of the Royal Society of Medicine* (Pathological Section), 1911, vol. iv, No. 6, p. 251.

The red corpuscles used in the complement fixation experiments were obtained by defibrinating the blood of a sheep, and then washing the corpuscles in three changes of saline solution. The hæmolytic serum was inactivated by heating it for half an hour at a temperature of 56° C. Fresh guinea-pig serum obtained from an animal killed on the day of the experiment was used as complement.

DESCRIPTION OF EXPERIMENTS AND RESULTS.

In the majority of these experiments the following method was employed: A series of progressive dilutions of the antigen was prepared in such a way that each successive tube contained half the amount of antigen contained in the tube immediately preceding it in the series. To every tube was then added a constant quantity of the antiserum. As a rule four such sets of tubes were prepared. One of these sets was set aside, that the appearance and amount of the precipitate in each tube might be observed and recorded. To the other sets was added complement in three different quantities or doses and, after one hour's incubation, a sufficient quantity of sheep corpuscles and hæmolytic serum. The set, to which no complement was added, provided a series of precipitates which varied not only in actual amount,

but also in the rate of formation and in the size of their constituent particles. The other three sets of tubes provided a roughly quantitative measure of the amount of complement bound by the various precipitates produced.

The progress of events in the tubes set aside for precipitation was as follows: Immediately after antigen and antibody had been mixed, a distinct turbidity was visible in two or three of the tubes of the series. The tubes which showed an instantaneous change were those tubes which contained antigen and antibody, in the proportions most favourable to the formation of precipitate. After five or ten minutes, or even sooner, very small particles became visible in the tubes which had been the first to become turbid. Below what may be termed the zone of optimal precipitation, that is to say, in the tubes which contained less than the optimal amount of antigen, turbidity in various degrees gave place gradually and progressively, tube by tube, to a condition in which the individual particles become visible to the naked eye. The particles in the tubes of the zone of maximal precipitation rapidly increased in size, and at the end of some twenty minutes it was usual to find that in two or three tubes large flocculi had separated, which rapidly fell to the bottom of the tube, leaving a perfectly clear supernatant fluid.

In such a series of tubes it was possible to recognize three zones: (1) A zone where the proportion of antigen to antibody was such as to produce rapid and complete precipitation. It may be assumed that in these tubes the antigen was able to rapidly precipitate the precipitable substance of the antiserum. (2) A zone in which the antibody was present in relative excess. In these tubes turbidity made its appearance slowly, and uniform turbidity slowly gave place to a condition in which small particles could be distinguished. In tubes in which a great excess of antibody was present the appearance of turbidity was still further delayed and, during the time of the experiment, the precipitation process did not go beyond the formation of an apparently homogeneous turbidity. (3) A zone where the antigen was present in relative excess. In these tubes the process of precipitation was slow. Turbidity appeared only after a considerable interval, and the appearance of separate particles was often delayed for some hours. A great excess of antigen entirely inhibited the formation of a precipitate.

Relative excess either of antigen or antibody was found to slow the process of precipitation, and if the excess was at all considerable, to lessen the amount of the precipitate.

The result of a typical experiment is given in Table I.

DESCRIPTION OF EXPERIMENT I AND NOTES ON THE COURSE OF
THE REACTION.

Column A represents the results of the precipitin experiment. Columns B, C, and D represent the results of the complement fixation experiments.

The tubes 1 to 11 in column A contained a bulk of 10 c.c., made up of 5 c.c. of the diluted goat serum (antigen) and 5 c.c. of a 1 in 10 dilution of rabbit and goat serum (antibody). The lower portion of each of these tubes was narrow and calibrated. The tubes were incubated for four hours at 37° C. and the progress of the precipitation was observed and recorded at regular intervals. *Immediately after mixing* turbidity appeared in tubes 2 and 3.

At the expiration of ten minutes flocculi were apparent in tubes 2 and 3, and tubes 1, 4, and 5 showed uniform turbidity. *After half an hour* large flocculi, which had formed in tubes 2, 3, and 4, had fallen to the bottom of the tubes and left a clear supernatant fluid. Flocculi had formed in tube 1, but had separated less completely. Small particles were visible in tube 5 and just visible in tube 6. In tubes 7 and 8 turbidity was present without visible particles. *After one hour* the first six tubes showed a deposit and a clear supernatant fluid. Tube 7 showed small particles suspended uniformly throughout its contents. In tubes 8, 9, and 10 a progressively diminishing opalescence was seen. *After four hours* all the tubes were centrifugalized and the actual amount of the deposited precipitate was read. The figures are recorded in column A.

In order to save unnecessary complexity the necessary control tubes have been omitted from the table. Control tubes containing the various dilutions of antigen without antiserum, and a tube containing a 1 in 10 dilution of the antiserum without antigen, were incubated for the same period (four hours) and remained absolutely clear.

The tubes in columns B, C, and D received each 0.5 c.c. of the various dilutions of goat serum (antigen) shown in the table. To all the tubes were then added 0.5 c.c. of a 1 in 10 dilution of antiserum and 0.5 c.c. of diluted guinea-pig complement. The tubes in column B received 0.05 c.c. guinea-pig serum diluted to 0.5 c.c., in column C 0.1 c.c. guinea-pig serum diluted to 0.5 c.c., and in column D 0.2 c.c. guinea-pig serum diluted to 0.5 c.c. All the tubes were then incubated for one hour at 37° C. in the same incubator as the tubes prepared

for the precipitin reaction. Both the precipitin and the complement fixation experiments were carried out with the same materials and as nearly as possible at the same time and under the same conditions. After one hour's incubation all the tubes received 0.5 c.c. of a 1 in 20 suspension of washed sheep corpuscles and 0.5 c.c. of a 1 in 500 dilution of hæmolysin (rabbit v. sheep). The tubes were then incubated for one hour, at the end of which time the results were read and recorded.

The fresh guinea-pig serum (complement) was separately titrated and its value determined; 0.02 of this serum diluted to 0.5 c.c. and added to 0.5 c.c. of a 1 in 500 dilution of hæmolysin, 0.5 c.c. of a 1 in 20 suspension of sheep cells and 1 c.c. of normal saline solution produced complete hæmolysis after one hour at 37° C. This was the smallest quantity of complement which produced complete hæmolysis acting in conjunction with 0.5 c.c. of a 1 in 500 dilution (two hæmolytic doses) of the hæmolytic serum. If we accept 0.02 c.c. as one lytic dose of the complement, column B represents the fixation of two and a half minimal lytic doses of complement, column C five minimal lytic doses, and column D ten minimal lytic doses. In the case of tube 6 of column D, nearly ten doses of complement were fixed.

A titration of the antiserum (rabbit v. goat) established the fact that this serum had a strong hæmolytic action on sheep corpuscles. This explains the fact that in this experiment complement fixation was not a more delicate indicator of the presence of a trace of antigen than the precipitin reaction.

It appears from this experiment that using a 1 in 10 dilution of antiserum the largest precipitate was obtained by adding an equal quantity of a 1 in 10 dilution of antigen. The greatest fixation of complement was obtained by adding to a 1 in 10 dilution of antiserum an equal quantity of a 1 in 320 dilution of antigen.

Remarks on Table I.

From a consideration of Table I it is evident that the proportions of antigen and antibody which produce the largest quantity of precipitate are not those which affect the greatest fixation of complement. In this case a mixture of a 1 in 10 dilution of antigen with a 1 in 10 dilution of antiserum produced a large precipitate, but did not fix two and a half minimal lytic doses of complement. Similar and consistent results were obtained by mixing a variety of antigens with the homologous antisera. It was determined that mixtures which

rapidly produced a large flocculent precipitate had little or no power to bind complement. If, however, the relative properties of antigen and antibody were such that the precipitate formed slowly, the mixture was found to have very distinct power to bind complement. Roughly speaking, the maximal fixation of complement corresponded to the mixture of antigen and antibody which at the expiration of half an hour showed the greatest uniform turbidity. If to a series of dilutions of the antigen was added a constant amount of antibody, two tubes

No Hæmolysis	
Trace "	
Slight "	
Half-Hæmolysed	
Strong or Marked Hæmolysis	
Almost Complete Hæmolysis	
Complete Hæmolysis	

Explanation of Scheme used to illustrate the Complement
Fixation Experiments.

Each tube in the original experiment is represented by a square in the table. A black square represents total hæmolysis or no fixation of complement. A white square represents a tube in which no hæmolysis has occurred, that is to say, a tube in which all the complement has been fixed.

could be picked out from the set—the one contained the proportions of antigen and antibody which produced the largest precipitate, the other the proportions of antigen and antibody most favourable to complement fixation. With constant antibody the amount of antigen necessary to produce the maximal fixation of complement was much smaller than the amount of antigen necessary to produce the largest amount of precipitate. In the experiment recorded in Table I it was found that thirty times as much antigen was required to produce the maximal precipitate as was necessary to produce the maximal fixation of complement.

The process which causes both precipitation and complement fixation is in all probability the aggregation of molecules present in the antiserum by the action of the antigen. When sufficient antigen is present aggregation takes place energetically and large flocculi are rapidly separated. These are the conditions favourable to the precipitation reaction. If a relatively smaller amount of antigen is present, the process of aggregation is slower and less complete. These are the conditions favourable to complement fixation. Under these conditions the individual particles which form the precipitate are extremely small, but the surface afforded by all the particles of such a precipitate must be much larger than that offered by a precipitate composed of large flocculi. It is indeed possible that there is a direct relationship between the surface area of the particles of the precipitate and the amount of complement absorbed.

In any case it is probable, for reasons that will appear from an experiment to be described later (Table II), that complement is fixed during the earliest stages of the formation of the precipitate, and that consequently the proportions of antigen and antibody which cause a very slow precipitation are those favourable to the fixation of complement. The fixation of complement seems to stand in close relationship with the rate of formation and the state of division of the precipitate. It can, in fact, be shown experimentally that either complement fixation or precipitation can be produced with the same antigen and the same antibody by merely varying the proportions of the mixture. It is obvious, then, that the fact that complement fixation and precipitation do not run a parallel course affords no evidence of the separate existence of two varieties of antibody, namely, precipitins and amboceptors, for the proportions which favour the rapid formation of a large precipitate are unfavourable to the fixation of complement, and the proportions which effect the best fixation of complement do not lead to the rapid formation of a precipitate.

It will be remembered that in Wassermann and Bruck's experiment, the fresh bacterial extract had the power of producing a precipitate and of fixing complement. The extract was then put aside, and at the time of the second experiment it was found to have lost its precipitating powers without loss of its complement-binding properties. This result, can, I think, be explained without postulating the existence of two different antibodies. It seems probable that during the interval between the first and second experiments a destruction of the specific antigen had taken place, and that at the time of the second experiment

the antigen content of the extract had been so far reduced that there was no longer sufficient to produce a visible precipitate. The conditions were, nevertheless, exactly those which have been shown to be favourable to the fixation of complement—namely, the presence of a relatively small quantity of antigen and a relatively large quantity of antibody.

TABLE I.

Dilutions of normal Goat Serum (antigen)	(A) 1 in 10 dilution of Rabbit v. Goat Serum (antibody) Amount of precipitate formed	1 in 10 dilution of Rabbit v. Goat Serum (antibody)		
		(B) 0.05 c. c. guinea-pig complement	(C) 0.1 c. c. guinea-pig complement	(D) 0.2 c. c. guinea-pig complement
(1) 1 in 10	90			
(2) 1 in 20	60			
(3) 1 in 40	40			
(4) 1 in 80	45			
(5) 1 in 160	40			
(6) 1 in 320	30			
(7) 1 in 640	10			
(8) 1 in 1280	5			
(9) 1 in 2560	Large trace			
(10) 1 in 5120	Trace			
(11) 1 in 10240	nil			

A similar explanation probably holds good for some experiments of Liefmann and of Friedberger. In Friedberger's experiments the anti-serum was heated for one hour at 67° C., and was then found to have lost its precipitating property, but not its complement-fixing property. The effect of heating on the antiserum was probably to render the

precipitation process slow and incomplete. These are the very conditions under which complement is best fixed. Liefmann heated the antigen-containing serum and destroyed its power of forming a precipitate without interfering with its complement-binding power. This experiment is probably capable of a similar explanation.

With the majority of antisera investigated it was found possible to demonstrate that the proportions which rapidly produced a precipitate did not effectively bind complement. There were, however, two exceptions in the series. In the case of these antisera, which were both exceptionally "weak," the precipitate was only very slowly formed, even when large quantities of antigen were used. It was not found possible in these cases to find proportions of antigen and antibody which produced a precipitate without binding complement. With these antisera, in which precipitation was always slight and slow, the amount of complement fixed was always proportional to the degree of turbidity produced. If, however, the antiserum was a "good" antiserum, it was always found possible to prepare a mixture of antigen and antibody in proportions which would rapidly precipitate but which did not fix complement.

EXPERIMENT II (TABLE II).

In this experiment the complement was added immediately and at varying periods after the mixtures of antigen and antibody were made.

Ten identical sets of antigen dilutions were prepared. To all the tubes was added the same amount of antiserum. One set was set aside for observations on the rate and degree of precipitation. Turbidity appeared immediately after mixing in the first four tubes. *After ten minutes* flocculi had appeared in the first three tubes, and tubes 4 and 5 showed turbidity. *After half an hour* large flocculi had separated, leaving a clear fluid in the first three tubes; tube 4 showed smaller flocculi, and tube 5 minute particles. Tube 6 showed marked uniform turbidity. Slight opalescence was present in 7 and 8. *After one hour* the precipitate had deposited in the first three tubes. In tubes 4, 5, and 6 smaller particles were still suspended in the fluid. Tubes 7 and 8 showed distinct turbidity, and a trace of opalescence could be detected in tube 9.

The other nine sets of tubes were treated as follows: To the first three sets complement in three different dilutions was added, and then to every tube the same amount of antibody. In the next three sets the antibody was added directly to the antigen, and the tubes were incubated

for half an hour. The complement was then added. In the next three sets the antigen and the antibody mixtures were incubated for one hour before the addition of complement. After the addition of complement each set of tubes was incubated for one hour, when a suitable mixture of corpuscles and hæmolytic serum was added. Each set was then incubated for a further period of one hour, at the end of which time the results were read and recorded. The experiment shows the result

TABLE II.
Antiserum 1 in 10 (Rabbit v Goat)

Dilution of Goat Serum (antigen)	Complement added immediately			after $\frac{1}{2}$ hr			after 1 hr.		
	Fresh Guinea-pig Serum 0.05	0.1	0.2	0.05	0.1	0.2	0.05	0.1	0.2
(1) 1 in 10									
(2) 1 in 20									
(3) 1 in 40									
(4) 1 in 80									
(5) 1 in 160									
(6) 1 in 320									
(7) 1 in 640									
(8) 1 in 1280									
(9) 1 in 2560									
(10) 1 in 5120									

of adding complement at various times after the mixture of the antigen with the antibody. A full set of control tubes was also prepared. In all these tubes complete hæmolysis occurred.

Remarks on Table II.

It is quite apparent that to obtain the greatest fixation the complement should be present from the time when antigen is mixed with antibody. After half an hour's preliminary incubation there was a

marked reduction in the complement-binding power of the mixtures. If one hour was allowed to elapse before complement was added very little fixation took place. As was anticipated, this loss of complement-binding power was more marked in the tubes in which the precipitation process was rapid. A comparison of the results obtained in tubes 4 to 8 in the various columns shows this point clearly. With the immediate addition of complement the maximum fixation occurred in tube 5, which contained a mixture of 1 in 160 antigen with 1 in 10 antibody. When half an hour had been allowed to pass before complement was added, the contents of tube 5 showed little power to fix complement, while tube 6, which contained a mixture of 1 in 320 antigen with 1 in 10 antibody, showed the best fixation of complement. The experiment bears out the view that complement is not fixed to any extent by the particles of a visible precipitate. It is probable that complement is fixed during the very earliest stage of the precipitation process at a time when the individual aggregates are so small that no turbidity is visible. When, on the other hand, the process has advanced to a stage at which visible precipitation occurs, the conditions necessary for the fixation of complement are no longer present.

EXPERIMENT III (TABLE III).

In Experiment III the amounts of precipitate which occur when varying quantities of antigen are added to a constant quantity of antibody have been measured and are recorded in Table III.

The table shows that the amount of the precipitate formed depends on the relative proportions of antigen and antibody present in the mixture. With a 1 in 5 dilution of antiserum the largest precipitate was obtained by adding a 1 in 8 dilution of antigen. With half the quantity of antiserum (1 in 10) the largest precipitate was obtained by adding a 1 in 16 dilution of antigen. The largest precipitate obtained with the 1 in 10 dilution of antiserum was about half the largest amount obtained by the use of twice as much antiserum (1 in 5 dilution). This is in accord with the view of Chapman and Welsh that the amount of the precipitate is proportional to the amount of antiserum present. One of the main results of all these experiments was to show that for any quantity of antiserum there exists a quantity of antigen which is capable, within a definite period of time, of producing the largest precipitate obtainable from such a quantity of antiserum. Table III also illustrates very clearly the inhibitory effect of relative

excess of antigen. This is plainly seen by comparing the figures in the two columns. A 1 in 4 antigen dilution, which constituted a marked antigen excess for an equal quantity of 1 in 10 antiserum, produced almost the largest precipitate of the series when mixed with a 1 in 5 dilution of antiserum.

TABLE III.

		5 c.c. dilutions of normal horse serum (antigen)		5 c.c. of a 1 in 5 dilution of rabbit v. horse serum (antibody)		5 c.c. of a 1 in 10 dilution of rabbit v. horse serum		5 c.c. normal saline solution
1	...	Undiluted serum	...	7.0	...	0.5	...	Clear
2	...	Diluted 1 in 2	...	14.0	...	3.0	...	"
3	...	" 1 in 4	...	19.0	...	6.5	...	"
4	...	" 1 in 8	...	20.0	...	8.0	...	"
5	...	" 1 in 16	...	11.0	...	12.0	...	"
6	...	" 1 in 32	...	6.0	...	6.0	...	"
7	...	" 1 in 64	...	4.0	...	2.5	...	"
8	...	" 1 in 128	...	3.0	...	1.5	...	"
9	...	" 1 in 256	...	2.0	...	0.75	...	"
10	...	" 1 in 512	...	1.0	...	0.5	...	"
11	...	" 1 in 1,024	...	0.75	...	Less than 0.5	...	"
12	...	5 c.c. normal saline solution	...	Clear	...	Clear	...	—

Three sets of dilutions of horse serum were prepared. To one set was added an equal quantity of a 1 in 5 dilution of antiserum, to another set was added a 1 in 10 dilution of the antiserum. The third set received an equal quantity of normal saline solution, and acted as an antigen control. The antiserum controls are shown in the tubes numbered 12. All the tubes were incubated for four hours at 37° C., and then placed overnight in the cold room. The tubes were then centrifugalized for one hour. The tubes ended in a narrow portion, which was calibrated in such a way that the amount of the precipitate could be accurately determined. The relative quantities of the precipitates in each tube are indicated by the figures.

EXPERIMENT IV (TABLE IV).

Experiment IV is designed to show more clearly the influence of excess of antigen on the resulting precipitate.

In the first two tubes the excess of antigen was so great that no trace of a precipitate appeared. In tube 3 the antigen was still present in relative excess although it contained only twice the optimal quantity of antigen. The effect of this excess was to render precipitation slow and imperfect. Opalescence only slowly made its appearance in this tube and after a considerable delay a few large flocculi became visible. Precipitation was even to the end of the experiment very incomplete and the contents of the tube remained permanently opalescent. It will be noticed that, if the proportions of antigen and antibody are approximately correct, precipitation rapidly proceeds by a process of aggregation

of particles to the formation of definite flocculi which fall to the bottom and leave a clear fluid. If there is a great excess either of antigen or antibody a condition of homogeneous turbidity or opalescence is the result.

The mixtures in which this slowing of precipitation is due to antibody excess have always very considerable power to bind complement. On the other hand, complement is not bound by mixtures in which antigen excess is responsible for imperfect precipitation. It will be seen from this and other tables that the precipitation reaction appears to be much more easily inhibited by excess of antigen than by excess of antibody. In Table IV, where the largest precipitate was produced by a 1 in 10 dilution, four times this quantity of antigen entirely inhibited the reaction, while a slight opalescence could be produced by the use of a one two-thousandth part of this quantity.

TABLE IV.—PRECIPITATION EXPERIMENT.

Dilutions of goat serum (antigen)				Rabbit v. goat serum (antibody) Diluted 1 in 10	
1	...	...	4 in 5	...	No deposit
2	...	...	2 in 5	...	No deposit
3	...	...	1 in 5	...	0.03
4	...	...	1 in 10	...	0.11
5	...	...	1 in 20	...	0.07
6	...	...	1 in 40	...	0.05
7	...	...	1 in 80	...	0.06
8	...	...	1 in 160	...	0.05
9	...	...	1 in 320	...	0.04
10	...	...	1 in 640	...	0.02
11	...	...	1 in 1,280	...	0.01
12	...	...	1 in 2,560	...	Large trace
13	...	...	1 in 5,120	...	Trace
14	...	...	1 in 10,240	...	No deposit; opalescent
15	...	...	1 in 20,480	...	No deposit; opalescent
16	...	...	1 in 40,960	...	No deposit; clear

The various amounts of antigen indicated in the table were mixed with a constant quantity of antiserum. The progress of precipitation was as follows: Immediately after mixing opalescence appeared in tubes 4 to 9. Small particles appeared after *five minutes* in tube 5, and shortly afterwards in tubes 4 and 6. After *ten minutes* big flocculi were present in tubes 4 and 5, and small flocculi in tube 6. After *half an hour* tubes 1 and 2 were still clear; tube 3 was opalescent; in 4, 5 and 6 large flocculi had fallen to the bottom of the tube, leaving a clear supernatant fluid; in 7 and 8 small particles were present; 9, 10 and 11 showed diminishing grades of uniform turbidity. After *two hours* tubes 1 and 2 were clear; in 3 a few flocculi had formed and fallen to the bottom, but the supernatant fluid was opalescent; in 4 to 11 the precipitate had settled to the bottom, leaving a clear supernatant fluid; 12 contained small particles still suspended in the fluid; 13 and 14 were distinctly opalescent, and 15 showed a trace of opalescence. After *three hours* the deposits were brought down by the centrifuge and their amounts recorded. Tubes 3, 14 and 15 remained opalescent after centrifugalization. Antigen and antibody controls were put up and remained clear.

EXPERIMENT V (TABLE V).

In this experiment an attempt has been made to show the effect of antigen excess in both precipitation and complement fixation. It will be seen that, as regards precipitation, the effect of the excess of antigen becomes more marked as the antiserum becomes more diluted. The complement fixation is more susceptible to the influence of antigen than the precipitation. There are two reasons for the inhibition of complement fixation by antigen excess which occur under two different

TABLE V.

Dilutions of Goat Serum (antigen)	Antiserum Rabbit v. Goat					
	Diluted 1 in 10		Diluted 1 in 20		Diluted 1 in 40	
	Precipitation	Complement Fixation	Precipitation	Complement Fixation	Precipitation	Complement Fixation
(1) 1 in 10	Large Deposit		Trace Deposit		No Precipitate	
(2) 1 in 20	" "		Small Deposit		" "	
(3) 1 in 40	" "		Deposit		Trace Deposit	
(4) 1 in 80	" "		"		Very small Deposit	
(5) 1 in 160	" "		"		" " "	
(6) 1 in 320	Deposit		"		" " "	

For the sake of simplicity the controls have been omitted from the table. All the usual control tubes were set up and were satisfactory. Six sets of antigen dilutions were put up. Three sets were reserved for the precipitation experiment and three sets for the complement fixation experiment.

sets of conditions. With great antigen excess no precipitation occurs; the mixture remains clear, and no complement is fixed. With a less quantity of antigen a large precipitate is formed. These are the conditions which, as has already been shown, are most favourable to the formation of a precipitate, but which do not favour the fixation of complement. If a long series of tubes is prepared in which various amounts of antigen are mixed with a constant amount of antibody,

a variety of zones may be differentiated. The largest amounts of antigen entirely inhibit both precipitation and complement fixation and the mixture remains clear. In the next zone precipitation is delayed and incomplete, and no complement is fixed. In the third zone precipitation is rapid and complete, but no complement is fixed. In the fourth zone precipitation begins again to be delayed and is less complete. These proportions represent *for precipitation* a slight antibody excess, nevertheless, it is in this zone that complement fixation appears and reaches its maximum. In the fifth zone precipitation is greatly delayed and is very incomplete. In this zone complement fixation is well marked, but nevertheless decreases tube by tube with the precipitate. In the sixth zone complement fixation can be demonstrated for a variable number of tubes after all evidence of precipitation has disappeared.

EXPERIMENT VI (TABLE VI).

This experiment is designed to show that similar inhibition phenomena occur in reactions between bacterial antigen and its corresponding antibody. It might be supposed that the inhibitory influence in the precipitate formation shown in the first three tubes of the experiment recorded in Table IV was due not so much to excess of specific antigen as to the presence of a relatively large amount of colloidal material or to a relatively small quantity of electrolytes. The antigen employed in this case was a distilled water extract of *Bacillus typhosus*. This extract contained very little proteid, and the fact that with this extract it was possible to demonstrate the influence of antigen excess is evidence that the inhibition is really due to an excess of the actual antigen.

In other respects Table VI shows the same points as Table III. The influence of the relative proportions of antigen and antibody on the amount of the precipitate is well shown. The method employed for measuring the amount of the precipitate was not, of course, extremely accurate, nevertheless the figures are sufficiently close to lend considerable support to the views of Chapman and Welsh. It will be seen from tube 2 that a mixture of 1 in 2 antigen with 1 in 5 antiserum produced a precipitate of 8. Tube 4 shows that a mixture of one quarter of the amount of antibody with one quarter of the amount of antigen produced one quarter of the amount of precipitate.

TABLE VI.

	5 c.c. dilutions of extract of <i>Bacillus typhosus</i> (antigen)	5 c.c. of a 1 in 5 dilution of anti- typhoid serum (antibody)	5 c.c. of a 1 in 20 dilution of antityphoid serum	5 c.c. normal saline solution (extract controls)
1 ...	Undiluted ...	15.0	... Very slight trace ...	Clear
2 ...	Diluted 1 in 2 ...	8.0	... 1.0 ...	..
3 ...	.. 1 in 4 ...	6.0	... 3.0 ...	..
4 ...	.. 1 in 8 ...	3.0	... 2.0 ...	..
5 ...	.. 1 in 16 ...	1.5	... 1.0 ...	..
6 ...	.. 1 in 32 ...	0.75	... 0.5 ...	..
7 ...	.. 1 in 64 ...	0.5	... 0.25 ...	..
<i>Serum controls—</i>				
8 ...	5 c.c. normal saline solution ...	Clear	... Clear ...	..

Three sets of dilutions of typhoid extract were prepared. To the first set was added an equal quantity of a 1 in 5 dilution of antityphoid serum; to the second set was added a 1 in 20 dilution; to the third set normal saline. The tubes were incubated for three hours, and then allowed to stand overnight in the cold room. On the next day the precipitate was brought down with a centrifuge and the amount of each precipitate was recorded.

ON THE QUANTITATIVE RELATIONS OF ANTIGEN AND ANTIBODY IN PRECIPITATION AND COMPLEMENT FIXATION EXPERIMENTS.

It will be seen from the experiments which have been described that the question of the relative proportions of antigen and antibody is one of the greatest importance in both the precipitation and the complement fixation reaction. Chapman and Welsh have shown that from any given quantity of antiserum a certain definite quantity of precipitate can be obtained. According to their view, the amount of precipitate is directly proportional to the amount of antiserum present. If to a constant quantity of antiserum are added various quantities of antigen the amount of the precipitate will in all cases be the same, provided that sufficient time be allowed for complete precipitation to take place. The larger quantities will rapidly precipitate the precipitable substance of the antiserum and the smaller quantities will precipitate it very slowly, but ultimately the same amount of precipitate will be produced.

In the experiments described in this paper precipitation has only been allowed to proceed for a limited time. When a short time only is allowed for precipitation, the results necessarily differ widely from the figures obtained by Chapman and Welsh. When only a short time is allowed, the amount of the precipitate depends on the relative proportions of antigen and antibody; that is to say, for any given quantity of antiserum a quantity of antigen can be found which is able rapidly to precipitate the precipitable substance in the given quantity of antiserum.

Such a mixture may be said to contain antigen and antibody in optimal or equivalent proportions. In such a mixture particles appear almost immediately, and after a few minutes large flocculi have aggregated together and fallen to the bottom of the tube, leaving a perfectly clear supernatant fluid in which apparently no further precipitate is formed. The whole process is under these conditions complete within a few minutes.

The importance of equivalent proportions of antigen and antibody for precipitation is fairly well shown in Tables III and VI.

If a series of quantities of antigen are mixed with a constant amount of antiserum and if the mixtures are centrifugalized after a few hours, it is found that for any given quantity of antiserum there exists a quantity of antigen which is able to produce the largest precipitate. If to a duplicate series of amounts of antigen there be added half the amount of antiserum, the largest precipitate will be half the amount of the largest precipitate of the first series, and it will be found in the tube which contains half the amount of antigen which produced the largest precipitate in the first series.

The amount of the precipitate can be shown to be proportional to the amount of antiserum (1) by adding any amount of antigen and waiting until precipitation is complete; (2) within a shorter period of time by adding the optimal amount of antigen.

In complement fixation the matter of equivalent proportions is of even greater importance, for it is essential that the experiment be carried out within a comparatively short period of time. It has been shown that the proportions of antigen and antibody which produce the most effective precipitation are not, as a rule, those which produce the greatest fixation of complement. For any quantity of antiserum it is generally possible to select two quantities of antigen, the one will produce the most rapid and complete precipitation, the other the greatest fixation of complement. The quantity which produces the best precipitation is many times greater than the quantity which produces the best fixation of complement. The fixation of complement is, in fact, dependent on two factors—the one is the amount of the precipitate, the other is the state of division of the particles of the precipitate, or rather the rate at which the precipitate is formed. The amount of complement fixed is proportional to the amount of the precipitate, provided that the precipitate is slowly formed. To show that the amount of complement fixed is proportional to the amount of antiserum present, it is obviously necessary to add to each quantity of antiserum the optimal quantity of antigen.

ON THE USE OF OPTIMAL PROPORTIONS IN THE PRACTICAL APPLICATION OF THE COMPLEMENT FIXATION TEST.

Experiment VII and Experiment VIII (Tables VII and VIII).

It has been shown that complement is best fixed when antigen and antibody are mixed in optimal proportions which have to be determined for any antigen and antiserum by actual experiment.

The experiments recorded in Tables VII and VIII show fairly clearly the inhibitory influence of the presence of a relative excess

TABLE VII.

Dilutions of Typhoid Extract	Antityphoid Serum diluted 1 in 5	Diluted 1 in 50	Diluted 1 in 100	Diluted 1 in 300	Normal Saline Solution Extract Controls
(1) 1 in 4					
(2) 1 in 8					
(3) 1 in 16					
(4) 1 in 32					
(5) 1 in 64					
(6) 1 in 128					
(7) 1 in 256					
(8) 1 in 512					
(9) 1 in 1024					
(Serum Controls)					
(10)					

The results shown in this table were obtained by mixing nine quantities of an extract of *Bacillus typhosus* with four quantities of an antityphoid serum. The extract and serum were the same as those used in the precipitation experiment recorded in Table VI. This experiment was carried out in the same manner as Experiment VIII. (See footnote to Table VIII.)

either of antigen or of antiserum. The complement fixation reaction is frequently used for the purpose of detecting the presence of antigen or antibody, for the purposes of diagnosis. If the material which is being investigated contains only a trace of the specific antigen, its presence can only be demonstrated by adding an approximately equivalent quantity of antiserum. For instance, it will be seen in Table VIII that the presence of antigen in an extract dilution of 1 in 1,280 could only be demonstrated by adding an antiserum dilution of 1 in 20; smaller and larger quantities of the antiserum failed to show any fixation of complement. Table VII shows that when the antiserum was diluted to 1 in 300 it was necessary to dilute the extract considerably (1 in 32) before any marked fixation of complement could be demonstrated.

Since the correct proportions must in every case be ascertained by direct experiments, it follows that it is advisable to use many quantities both of the antigen and of the antiserum if it is desired to detect the presence of a minute trace of either.

The complement fixation method is often used for the quantitative estimation both of antigen and of antibody. It has been recommended as a method of determining the "strength" of antimeningococcus serum, and efforts have been made to devise a method for giving a quantitative value to the results of the Wassermann reaction. In one method a constant quantity of the antigen is selected and titrated with falling quantities of antiserum. The probable result of this method is seen in Table VII. If a 1 in 8 dilution of extract has been selected as the constant quantity of antigen, the limit of the antiserum is reached at a dilution of 1 in 100. Had a 1 in 32 dilution of extract been selected, the limit would not have been reached until the antiserum had been diluted to 1 in 300. The apparent titre of the antiserum is in fact determined by the quantity of extract (antigen) selected.

The other method is to keep the dilution of antiserum constant, and to use falling dilutions of the extract. The results to be expected from this method are shown in Table VIII.

It will be seen from this experiment that if an antiserum dilution of 1 in 5 was taken, the extract could be diluted to 1 in 320, but if the antiserum dilution selected was 1 in 20 the extract could be diluted to 1 in 1,280. The result arrived at is that the serum dilution 1 in 20 is stronger than the serum dilution 1 in 5. If we suppose that the 1 in 5 serum dilution represents a constant quantity of an antiserum A, and that the 1 in 20 dilution represents an equal quantity of another

antiserum, we arrive by this method at a result which shows that antiserum B is stronger than antiserum A. It is equally obvious that the strength of the extract could not have been determined either by taking a constant amount of extract and falling quantities of antiserum or by taking a constant amount of antiserum and titrating it with falling quantities of extract.

TABLE VIII.

Dilutions Typhoid Extract.	Dilutions of Antityphoid Serum							Normal Saline Extract Control.
	1 in 5	1 in 10	1 in 20	1 in 40	1 in 80	1 in 160	1 in 320	
(1) 1 in 5								
(2) 1 in 10								
(3) 1 in 20								
(4) 1 in 40								
(5) 1 in 80								
(6) 1 in 160								
(7) 1 in 320								
(8) 1 in 640								
(9) 1 in 1280								
(10) 1 in 2560								
Serum Controls								

In this experiment ten dilutions of a typhoid extract and seven dilutions of an antityphoid serum were used. Every dilution of extract was allowed to interact with every dilution of antiserum. Each tube contained 0.5 c.c. of an antiserum dilution, 0.5 c.c. of an extract dilution, and 0.5 c.c. fresh guinea-pig serum in a dilution of 1 in 10. After incubation for one hour at 37° C. there were added 0.5 c.c. of a 1 in 20 suspension of sheep corpuscles and 0.5 c.c. of a 1 in 400 dilution of hæmolytic serum (rabbit v. sheep). The results were read after a second period of incubation for two hours. The extract and serum were different from those used in Experiment VII.

As far as these experiments go it appears that if a constant quantity of extract be added to varying quantities of antiserum valid *relative* differences can be obtained between two given sera. If, however, varying quantities of extract be added to a constant quantity of antiserum a greatly diluted antiserum can be shown to be stronger than the one which has been less diluted.

To make an absolute quantitative estimation either of the antibody content of an antiserum or of the antigen content of an extract it is necessary to titrate falling quantities of antiserum with falling quantities of the extract.

If it be desired to determine the absolute complement-binding power of any quantity of antiserum it is essential to mix with it the equivalent amount of extract. When the correct proportions have been determined the amount of complement bound may be estimated by the use of various amounts of complement. The value of using various amounts of complement as a quantitative method was pointed out by Muir and Martin (1906). The actual complement-binding power of any amount of antiserum can, however, only be determined if it is mixed with the correct amount of extract.

ON THE CAUSE OF THE INHIBITION OF THE REACTION BY RELATIVE EXCESS OF EITHER ANTIGEN OR ANTIBODY.

No theory of the cause of complement fixation can be considered satisfactory which does not explain the inhibition effects produced by relative excess of either antigen or antibody. It seems rather a difficult matter to interpret these effects on the basis of the amboceptor hypothesis. The only possible explanation would be similar to that offered by Neisser and Wechsberg (1901) in their paper on bacteriolysis.

It will be remembered that in their experiments, when a large quantity of antiserum was present no bacteriolysis took place. Neisser and Wechsberg put forward the view that under these circumstances the mixture contained a large excess of free amboceptors, that is to say, amboceptors which had not become attached to the bacilli. They assumed that these free amboceptors took up a large quantity of the complement, with the result that there was not sufficient complement for the amboceptors which had attached themselves to the bacilli. It does not seem easy to apply this highly theoretical explanation to the effects shown in Tables VII and VIII. If it is assumed that in this case the complement is deviated by the excess of free amboceptors, it

is also necessary to assume that the complement is subsequently liberated from the free amboceptors, to be taken up by the combination of hæmolytic amboceptors and red corpuscles. The amboceptor hypothesis, moreover, leaves us without any explanation of the inhibition produced by a relative excess of extract.

Now it has been shown that when either antiserum or antigen is present in relative excess, precipitation is very greatly delayed. Under these conditions the aggregation process necessary to complement fixation does not occur, or at any rate does not occur within the hour allowed for the reaction. The precipitation theory affords a much better explanation of the excess effects than the amboceptor theory. As has already been pointed out, excess of antigen interferes with the fixation of complement in two ways. If the excess is very great no precipitate is formed, and no complement fixed. With less quantities of antigen a stage can, as a rule, be demonstrated, in which the precipitation occurs so rapidly that the early stages, during which complement is probably fixed, are passed through too rapidly to permit of effective fixation taking place.

THE QUANTITATIVE ESTIMATION OF SERUM IN THE WASSERMANN REACTION (TABLE IX).

This experiment has been recorded in order to show that excess effects can be demonstrated with a positive syphilitic serum, and an extract of syphilitic liver. It will be seen that an accurate quantitative result cannot be obtained either by titrating a constant quantity of extract with falling amounts of serum, or by keeping the serum constant, and varying the quantities of extract.

The point raised is fairly well shown in Table IX. If a dilution of 1 in 10 extract had been selected as the constant quantity, it would have been found that a "complete reaction" could be obtained after the serum had been diluted to 1 in 20. If, however, half the quantity of extract had been selected, a practically complete reaction would have been obtained after the serum had been diluted to 1 in 40. It will be seen that the extent to which the serum can be diluted depends on the dose of extract selected.

The table also shows the result of using a constant amount of serum and falling amounts of extract. If we take two dilutions of serum, 1 in 5 and 1 in 20, and titrate each quantity with various quantities of the extract, we find that, using the 1 in 20 serum

dilution, the extract can be diluted further than it can be if a 1 in 5 dilution is used.

If these two dilutions of serum, 1 in 5 and 1 in 20, be taken as representing two separate sera, which are to be investigated, and if a constant quantity of each is titrated with falling quantities of extract, it is obvious that an incorrect result will be obtained.

TABLE IX

Dilutions of Syphilitic Serum	Dilutions of Liver Extract.				Saline Serum Controls.
	1 in 10	1 in 20	1 in 40	1 in 80	
1 in 5					
1 in 10					
1 in 20					
1 in 40					
1 in 80					
1 in 160					
Saline Extract Controls					

The materials used for this experiment were the serum from a syphilitic patient and an alcoholic extract prepared from the liver of a syphilitic foetus. Each tube (excepting, of course, the control tubes) contained 0.5 c.c. of diluted extract, 0.5 c.c. of diluted serum, and 0.5 c.c. of guinea-pig serum diluted 1 in 10. After one hour's incubation at 37° C. there were added 0.5 c.c. of a 1 in 20 suspension of sheep corpuscles and 0.5 c.c. of a 1 in 500 dilution of hæmolytic serum. The results were read after a second period of incubation lasting for one hour.

To obtain an accurate quantitative result, various quantities of extract must be mixed with various quantities of serum. When the optimal proportions have been ascertained the actual amount of complement fixed can be determined by the use of various quantities of complement.

Apart from the question of attempting to make the Wassermann

reaction quantitative, it is certainly advantageous at all times to use a series of dilutions. It will be seen from the table that a 1 in 40 dilution of serum gives a marked fixation of complement with an extract dilution of 1 in 20, but only partial fixation with an extract dilution of 1 in 10.

EXPERIMENT X (TABLE X).

This experiment is similar to those shown in Tables VII and VIII. Seven quantities of typhoid extract and six quantities of antityphoid serum were used. The experiment was set up in such a way that every quantity of extract was mixed with every quantity of antiserum. There resulted forty-two mixtures, each containing different proportions of antigen and antibody. Each of these mixtures was represented by three tubes, each of which received a different dose of complement. A quantitative measure of the amount of complement bound by each of the mixtures was thus attempted. The value of the complement was determined by a separate experiment, and the minimal hæmolytic dose of the guinea-pig serum was found to be 0.0125 c.c. The method is of course only roughly quantitative, for it must not be assumed that 0.025 c.c. has *exactly* twice the complement value of 0.0125 c.c. of the guinea-pig serum. The method was nevertheless sufficiently accurate to give comparative results of some interest.

In the first three columns corresponding to the larger amounts of antiserum the quantities of complement used were 0.2 c.c., 0.1 c.c., and 0.05 c.c. In the three last columns corresponding to the smaller amounts of antiserum the amounts of complement were 0.1 c.c., 0.05 c.c., and 0.025 c.c. The experiment was performed in essentially the same way as in the case of the other experiments described in this paper. The extract dilutions were first prepared, the complement was added next, and finally the antiserum. After one hour's incubation at 37° C. the blood and hæmolyisin were added. After a further period of incubation (two hours) the results were read. As in all the other experiments, each of the five ingredients was added in a bulk of 0.5 c.c., and the total quantity of fluid in each tube was thus 2.5 c.c.

It will be seen that the larger quantities of extract spontaneously bound the smallest quantity of complement used. The extent to which this spontaneous binding occurred is shown in the extract control column, and as a matter of fact, the result of the experiment was not in the least obscured.

Remarks on Table X.—The Relation between the Amount of Antiserum and the Amount of Complement Fixed.

This experiment demonstrates once more the importance of optimal proportions of antigen and of antibody.

						Extract
0.0125 c.c.	antiserum	bound	most	complement	when mixed with	0.05 c.c.
0.00625	"	"	"	"	"	0.05 or 0.025 c.c.
0.003125	"	"	"	"	"	0.025 or 0.0125 c.c.
0.0015625	"	"	"	"	"	0.0125 c.c.
0.00078125	"	"	"	"	"	0.00625 c.c.
0.000390625	"	"	"	"	"	0.003125 c.c.

That is to say, as the amount of antiserum was diminished the amount of extract necessary to give the best results also diminished. It is, in fact, quite impossible to estimate the antibody content of any given amount of serum, unless the correct amount of antigen is used. With the data provided by Table X it is, however, possible to form a rough estimation of the amount of complement bound by each quantity of antiserum.

By selecting (in Table X) the most effective dose of extract for each quantity of antiserum it is possible to roughly determine the complement bound in each case.

It will be seen that the amount of complement bound is approximately proportional to the amount of antiserum. It is probable that when antiserum and antigen are mixed in the proportions most favourable to complement fixation that the amount of complement fixed is in proportion to the amount of antiserum. This is in close agreement with the results of the precipitin experiments, in which it is shown that the amount of precipitate is in proportion to the amount of antiserum, provided that antigen and antiserum are mixed in equivalent proportions.

THE RELATIVE ADVANTAGES OF THE PRECIPITATION AND COMPLEMENT FIXATION METHODS.

From the time of its first introduction by Neisser and Sachs, the complement fixation method has been generally considered to possess several advantages over the precipitation method. It is, in the first place, the more delicate test for the presence of a minute trace of antigen. It has also been pointed out that the results obtained are more easily observed, for the absence of hæmolysis is more easily

Antitypho

[illegible]

TABLE X—(continued).

[illegible]

determined than the presence of a faint trace of opalescence or turbidity. There is, moreover, the great advantage that a perfectly clear solution of antigen is not an absolute essential. Besides these merits, which are sufficiently well known, the complement fixation method is superior to the precipitation method, in that it allows of a finer differentiation of nearly allied bacteria, and of the sera of nearly allied animals. With both methods group reactions not infrequently cause considerable difficulty, but with the complement fixation method there are two ways in which these difficulties can to a great extent be overcome.

The first of these ways consists in diluting the antiserum until a dilution is obtained which no longer gives the group reaction. This procedure is often impossible with the precipitation test, for the precipitate is derived from the antiserum, and if the antiserum is greatly diluted the precipitate is so reduced that it is no longer visible. For practical purposes differentiation can only be obtained with the precipitation method by greatly diluting the antigen. The complement fixation method permits extreme dilution both of antigen and antiserum.

The other method, by which the group reaction may be eliminated, is by increasing the amount of complement. For instance, a mixture of human serum with the antiserum derived from a rabbit which has been injected with human serum absorbs complement, but a mixture of monkey serum with the same antiserum (rabbit v. man) also absorbs complement. By using various amounts of complement, it can be shown that a mixture of human serum with rabbit v. man antiserum absorbs more complement than a mixture of monkey serum with rabbit v. man antiserum.

It is also frequently possible to eliminate a group reaction by using larger quantities of the hæmolytic antiserum.

Examples of the effect of varying the quantities of antiserum and of complement are given in the following tables.

Remarks on Tables XI, XII and XIII.

For these experiments rabbits were immunized with normal human serum and with normal monkey serum (*Macacus rhesus*). The antisera obtained were not very powerful, but served, nevertheless, to demonstrate the points which have been mentioned.

Table VIII shows the results of a precipitation experiment. Both

sera produced marked precipitates with the 1 in 2½ and the 1 in 10 dilutions of the antihuman serum. The precipitates produced by the human serum were somewhat more marked than those produced by the monkey serum, but the difference was insignificant. When the antiserum was used in a dilution of 1 in 40 precipitation practically ceased. In Table XII a complement fixation experiment is shown. In this case, too, the 1 in 10 and 1 in 20 dilutions of the antisera failed to bring out any striking difference between human serum and monkey serum. When, however, the antisera were diluted to 1 in 40 and 1 in 80 a perfectly satisfactory differentiation was obtained.

TABLE XI.—PRECIPITATION EXPERIMENT.

	Dilution of normal human serum	Rabbit v. man serum diluted			
		1 in 2½	1 in 10	1 in 40	1 in 160
1	1 in 100	Large deposit	Deposit	Slight deposit	Nil
2	1 in 200	"	"	Trace	"
3	1 in 400	"	"	Nil	"
4	1 in 800	"	"	"	"
5	1 in 1,600	Small deposit	Trace	"	"
6	1 in 3,200	Trace	Nil	"	"
7	1 in 6,400	Nil	"	"	"

	Dilution of monkey serum (<i>Macacus chasus</i>)	Rabbit v. man serum diluted			
		1 in 2½	1 in 10	1 in 40	1 in 160
1	1 in 100	Large deposit	Deposit	"	"
2	1 in 200	"	"	"	"
3	1 in 400	"	"	"	"
4	1 in 800	Small deposit	Trace	"	"
5	1 in 1,600	Trace	Nil	"	"
6	1 in 3,200	Nil	"	"	"
7	1 in 6,400	"	"	"	"

Various dilutions of normal human serum and of monkey serum were mixed with antihuman serum in four dilutions. The resulting precipitates are indicated in the table.

In the experiment recorded in Table XIII the same antisera were employed as of Table XII. In Table XII it was shown that the 1 in 10 dilutions of the antisera showed no distinction between human serum and monkey serum. In Table XII the usual quantity of complement, 0.5 c.c. of a 1 in 10 dilution of guinea-pig serum, was employed. In Table XIII twice this quantity was used, with the result that a marked differentiation was obtained.

When it is desired to differentiate between the sera of closely allied animals, the complement fixation method is to be preferred to the precipitation method. The results of the complement fixation experiment are, to a great extent, under the control of the worker. In the first place, it is possible, by varying the amount of complement, to

estimate quantitatively the complement-binding power of various antigen and antibody mixtures. It is also possible to control the degree of hæmolysis by varying the amount of the hæmolytic serum used, and by varying the time of the second period of incubation during which the hæmolysis takes place. These methods of modifying the result are often extremely useful when a fine degree of differentiation is required.

The great advantage, however, which the complement fixation method possesses is that it permits of the employment of many dilutions of antiserum. With the precipitation method the only possible way is to take a fixed dose of antiserum, and titrate it with falling doses of the antigen. The complement fixation method permits the use of various quantities, not only of antigen but of antiserum. By selecting a suitable quantity of antiserum a group reaction can be, as a rule, eliminated.

DISCUSSION OF RESULTS.

The evidence for the separate identity of complement-binding antibodies and precipitins has been given at some length in the early portion of this paper. There is only need to take up the principal arguments and examine them in relation to the present experiments.

(1) *Many instances have been recorded in which there has appeared to be no relation between the amount of precipitate formed and the amount of complement fixed: (a) either a large precipitate has formed and no complement has been fixed, (b) or a slight opalescence has been observed or perhaps no precipitate at all, but complement has been very efficiently fixed.*

Now it has been shown that by taking any one antigen and any one antibody, and by simply varying their relative proportions, it is possible to produce either precipitation without complement fixation, or complement fixation without precipitation. If the proportions are so adjusted that the precipitate is rapidly formed, little or no complement is bound, but if the proportions are such that precipitation is slow and incomplete, nothing more than a slight opalescence may appear, even after the lapse of hours; complement is, nevertheless, efficiently fixed. The amount of complement absorbed is conditioned by two factors—amount of precipitate formed and the rate of its formation. If the precipitate is very rapidly formed no complement is fixed, but if the formation of a precipitate is sufficiently slow the amount of complement absorbed is always found to be proportional

TABLE XII.

COMPLEMENT FIXATION EXPERIMENT

Dilutions of Normal Human Serum	Dilutions of Antiserum Rabbit v. Man			
	1 in 10	1 in 20	1 in 40	1 in 80
(1) 1 in 200				
(2) 1 in 400				
(3) 1 in 800				
(4) 1 in 1600				
(5) 1 in 3200				
Dilutions of Normal Monkey Serum (<i>Macacus Rhesus</i>)				
(1) 1 in 200				
(2) 1 in 400				
(3) 1 in 800				
(4) 1 in 1600				
(5) 1 in 3200				
Dilutions of Normal Human Serum	Dilutions of Antiserum Rabbit v. Monkey			
	1 in 10	1 in 20	1 in 40	1 in 80
(1) 1 in 200				
(2) 1 in 400				
(3) 1 in 800				
(4) 1 in 1600				
(5) 1 in 3200				
Dilutions of Normal Monkey Serum (<i>Macacus Rhesus</i>)				
(1) 1 in 200				
(2) 1 in 400				
(3) 1 in 800				
(4) 1 in 1600				
(5) 1 in 3200				

Various dilutions of normal human serum and normal monkey serum (*Macacus rhesus*) were mixed with various dilutions of rabbit v. man and rabbit v. monkey antisera. 0.5 c.c. of the antigen dilution was mixed with 0.5 c.c. of the antiserum dilution and 0.5 c.c. of a 1 in 10 dilution of fresh guinea-pig serum. This 1 in 10 dilution of guinea-pig serum was shown to contain two hæmolytic doses of complement. After incubation for one hour sheep corpuscles and hæmolytic serum were added, and the tubes were incubated for a further period of one hour. The usual controls, designed to show that none of the sera used had an anti-complementary action, were also done, but are not shown in the table.

to the amount of the precipitate. In the case of most antisera it is possible to demonstrate either marked precipitation with little or no complement fixation, or complement fixation with little or no precipitation. Some antisera, however, are in all dilutions and proportions very slowly precipitated, and in these cases the amount of precipitate is always proportional to the amount of complement fixed. The fact that complement fixation occurs without the formation of a visible precipitate, and a bulky precipitate may form without complement being fixed, cannot be accepted as evidence that the two reactions depend on the existence of two separate antibodies.

(2) *Complement fixation can be demonstrated in mixtures which contain so minute a quantity of antigen that no trace of a precipitate can be detected.*

That a trace of antigen can be detected by the complement fixation method when no trace of a precipitate can be seen is almost universally admitted. This fact, however, is no evidence that precipitation and complement fixation are independent phenomena. When only a trace of antigen is present the precipitation process is incomplete and extraordinarily slow. Now it has been shown that it is during the very earliest stages of the reaction between antigen and antibody that complement is best fixed. There is, indeed, reason for thinking (Table II) that the greater part of the complement is fixed during the earliest period of the reaction before even a trace of opalescence makes its appearance.

(3) *By heating the antiserum or the antigen containing serum, or by merely storing an extract, the power of forming a precipitate is destroyed while the power of binding complement is retained.*

A similar explanation to that offered in the last paragraph may reasonably be given. When a bacillary extract is stored away a gradual loss of specific antigen may be supposed to occur. The old extract is no longer able to produce rapidly an obvious precipitate, but is only able to precipitate the antiserum slowly and incompletely. These are just the conditions which favour complement fixation. Heat probably acts by altering either antigen or antibody, so that precipitation is slower and less complete, and consequently favourable to the fixation of complement.

(4) *The serum of an animal during the process of immunization develops complement-binding properties earlier than the power of forming a precipitate.*

This is exactly the result that might be anticipated. The more

Dean: *Fixation of Complement*TABLE XIII
COMPLEMENT FIXATION EXPERIMENT

Dilutions of Normal Human Serum	Antiserum Rabbit v. Man Diluted 1 in 10	Antiserum Rabbit v. Monkey Diluted 1 in 10
(1) 1 in 100		
(2) 1 in 200		
(3) 1 in 400		
(4) 1 in 800		
(5) 1 in 1600		
(6) 1 in 3200		
(7) 1 in 6400		
Dilutions of Normal Monkey Serum (Macacus Rhesus)		
1. 1 in 100		
(2) 1 in 200		
(3) 1 in 400		
(4) 1 in 800		
(5) 1 in 1600		
(6) 1 in 3200		
(7) 1 in 6400		

In this experiment all the materials used were exactly the same as those used in Experiment XII. The only difference is that exactly twice as much complement was used. In Experiment XII the usual dose of complement, guinea-pig serum in a dilution of 1 in 10, was used. In Experiment XIII a 1 in 5 dilution of guinea-pig serum was used and complete differentiation obtained.

delicate reaction is, as might be supposed, the first to make its appearance. The slight aggregation of particles which follows the mixture of antigen and "weak" antiserum is not sufficient to produce a visible precipitate, but can be demonstrated by the complement fixation reaction.

It should also be remembered that the common method of determining the titre of a precipitin serum is to take a constant amount of the antiserum, usually quite a large amount, and to test it with falling quantities of the antigen. For determining the complement-binding value of a serum the reverse method is commonly used—that to say, a constant amount of antigen is mixed with falling quantities of antiserum. In such experiments the result is dependent on the relative proportions of antigen and antibody. If the complement-binding and precipitate-forming properties are compared, the dilutions, relative proportions, and the times allowed for the reactions are usually different. Under such conditions it is not surprising that the complement binding properties are earlier demonstrable than the precipitating properties or the reverse. The results are dependent on the experimental conditions.

The fact that Chapman and Welsh have been able to prove that the precipitate is derived from the antiserum has an important bearing on the relation between precipitate formation and complement fixation. When it was held that the precipitate was derived from the antigen it was difficult to see how the precipitate obtained from an exceedingly minute trace of antigen could be effective in complement fixation. Chapman and Welsh have shown that it is the antigen which precipitates the antiserum, and that a very minute trace of antigen is able, if sufficient time is allowed, to precipitate a relatively enormous precipitate from the antiserum. The action of the antigen is to induce an aggregation of the molecules of the antiserum, and the earlier stages of this process produce the effective conditions for complement fixation. It is probable that the researches of Chapman and Welsh will prove of value in helping to explain the mechanism of other serum reactions.

It has been shown that the greater part of the complement is bound at a very early stage in this aggregation process. Indeed, after a visible opalescence has once formed it seems that very little complement is taken up. The fixing of complement is probably an adsorption process for which the optimal conditions are afforded during the earlier stages of precipitation. This view is supported by the results obtained in Experiment II.

Chapman and Welsh have shown that the amount of precipitation which can be obtained from any given quantity of antiserum is a constant quantity which is independent of the quantity of antigen which may be present. This result is obtained if sufficient time be allowed for complete precipitation. The rate, however, at which precipitation takes place is closely dependent on the amount of antigen present. If precipitation is interrupted after a few hours, the amount of the precipitate formed bears a direct relationship to the amount of antigen present.

In complement fixation experiments the reaction which takes place in the first hour which follows the mixture of antigen and antibody is alone of importance. If a constant quantity of antiserum be mixed with a series of quantities of antigen, a quantity of antigen can be selected which gives the largest precipitate within the time-limit of the experiment. Such a quantity of antigen may be called the optimal quantity of antigen, because it is the quantity which acts the most rapidly and efficiently on the selected quantity of antiserum. Such quantities of antigen and antiserum may be said to be the optimal proportions for precipitation.

The relation of the amount of complement fixed to the amount of precipitate formed is complicated by the influence of another factor, the rate at which precipitation takes place. The conditions under which complement fixation takes place are those under which the presence of a relative excess of antiserum produces slow and incomplete precipitation. Provided that the mixture contains a sufficient excess of antiserum, it is true that the amount of complement fixed is proportional to the amount of precipitate formed, but if the proportions are such that precipitation is rapid, then little or no complement is fixed. These experiments offer an explanation of the contradictory results which have been published, for the question as to whether a relation can be demonstrated between the amount of precipitate formed and the amount of complement fixed depends on the relative proportions of antigen and antibody.

The proportions, in fact, which favour rapid and complete precipitation are positively unfavourable to complement fixation. On the other hand, there is a definite relation between the proportions most favourable to precipitation and those most favourable to complement fixation. If a constant amount of antiserum be taken it will, in the majority of cases, be found that the amount of antigen which will produce the best complement fixation is many times less than the amount

necessary to produce the largest precipitate. The reason why the two reactions do not run a parallel course is not because they are caused by two different sets of antibodies, precipitins and amboceptors, but because they represent two phases or two stages of the same reaction, and it is not possible to demonstrate both stages under the same conditions. A flocculent precipitate represents the complete and final stage of a change which can be recognized in its earliest and incomplete stage by means of the complement fixation method.

SUMMARY AND CONCLUSIONS.

(1) The mixture of equivalent proportions of antigen and antibody results in the rapid formation of the largest precipitate which can be obtained from any given quantity of antiserum.

(2) When the mixture contains an excess either of antigen or antibody, precipitation is delayed.

(3) Great excess either of antigen or of antibody entirely inhibits the formation of a precipitate.

(4) When antigen and antibody are mixed in the proportions which rapidly form a large precipitate very little or no complement is fixed. There is, under these conditions, no relation between the amount of precipitate formed and the amount of complement fixed.

(5) In mixtures in which precipitation is delayed by a relative excess of antibody there is a marked fixation of complement.

(6) The most marked fixation is obtained by mixing definite proportions of antigen and antibody.

(7) If a constant quantity of antibody is added to a series of quantities of antigen, two quantities of antigen can be selected. The one quantity is that which produces the largest precipitate, the other quantity is that which produces the greatest fixation of complement. The mixture which produces the greatest fixation of complement always contains considerably less antigen than the mixture in which the largest precipitate is produced.

(8) Relative excess either of antigen or antibody results in a decrease in the amount of complement which is fixed. If the mixture contains antigen in excess of the amount most favourable to the fixation of complement, precipitation is more rapid, but less complement is fixed. With a further increase in the relative amount of antigen precipitation is also inhibited. If, however, the amount of antigen is less than that which produces the best fixation of complement there is a diminution

not only in the amount of complement fixed, but also in the amount of the precipitate. With a further decrease of antigen both reactions steadily decrease until a dilution is reached at which visible precipitation ceases while a certain degree of complement fixation remains.

(9) In mixtures in which precipitation is sufficiently delayed by relative antibody excess the amount of complement fixed bears a direct relationship to the amount of precipitate formed.

(10) Provided that antiserum and antigen are mixed in optimal proportions, the amount of complement fixed is proportional to the amount of antiserum present.

(11) In mixtures in which delayed and incomplete precipitation is due to relative excess of antigen complement is not fixed.

(12) *It seems probable that complement is fixed during the earliest stages of the aggregation process by which the precipitate is formed. After a visible precipitate has formed very little complement is absorbed. In mixtures which contain a relative excess of antibody precipitation is incomplete and delayed, and the earlier stages occupy sufficient time to allow the complement to be bound.*

(13) To ensure the greatest possible fixation, the complement should be present from the time when antigen is mixed with antibody. If an interval is allowed to elapse before complement is added, very much less fixation takes place.

(14) *The fact that precipitation has been observed without complement fixation, and complement fixation without precipitation, is not sufficient evidence that two separate antibodies exist, for by adding different quantities of antigen to a constant amount of the same antiserum it is possible to demonstrate precipitation without complement fixation or complement fixation without precipitation.*

(15) Complement fixation and precipitation are two methods by which we are enabled to measure the same change which represents the aggregation of the particles of an antiserum by the homologous antigen.

(16) When the complement fixation method is used for the purpose of detecting the presence of antigen it is necessary to use various dilutions of the antiserum. Unless an approximately equivalent amount of antiserum is employed it is always possible that complement fixation may be inhibited by a relative excess either of antigen or of antibody.

(17) In order to make a quantitative determination either of antigen or of antibody it is necessary to titrate various amounts of antigen with various amounts of antibody, for unless antigen and antibody are

present in equivalent proportions the greatest fixation of complement cannot be obtained. After the equivalent proportions have been ascertained the actual amount of complement fixed can be demonstrated by the use of various amounts of guinea-pig serum, of which the complement content has been determined.

(18) The complement fixation method is more convenient than the precipitation method for the purpose of differentiating between nearly allied varieties of bacteria and the sera of nearly allied animals. Complete differentiation can frequently be only obtained by a dilution of the antiserum which is too weak to produce a visible precipitate. Another method of securing differentiation is by the use of various doses of complement.

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A Case of Ulcerative Endocarditis produced by the Pneumococcus in a Child, aged 3.

By H. R. DEAN.

THE patient was a female child, aged 3. She was said to have been always a weakly child. The illness began with vomiting on January 14. On the next day she was drowsy, but screamed if roused. On January 16 she was admitted to the Victoria Hospital for Children under the care of Dr. A. C. D. Firth. The typical signs of meningitis were present. The patient died on January 17.

Post-mortem examination: The body was poorly nourished. A well-marked purpuric eruption was present on the skin of the thighs and shins. The individual petechiæ were small, round, well defined, and of a purple colour. The pericardial sac contained a little clear fluid. The heart was large. Both ventricles were dilated and hypertrophied. Both auricles contained laminated clot. The clot contained in the right auricular appendix was adherent to the wall of the chamber. The right ventricle contained a large laminated clot which was adherent to the anterior wall of the ventricle immediately below the tricuspid valve. On removal of the clot an area of superficial ulceration was found. Numerous vegetations were present in the space between the infundibular segment of the tricuspid valve and the wall of the ventricle. There were no vegetations on the internal surface of the segments of the tricuspid valve. The mitral valve was thickened. One large and several smaller patches of granulations were found on the anterior segment of the mitral valve. The left ventricle contained a laminated clot which was adherent to the mitral valve. The aortic and pulmonary valves were normal. The lungs were extremely congested and œdematous, but there was no sign of consolidation. The peritoneum was reddened and injected throughout. The liver was large and on section presented a typical fatty nutmeg appearance.

2 Dean: *Ulcerative Endocarditis produced by Pneumococcus*

The spleen was large and firm. The kidneys were of normal size, of a tough consistence and a deep red colour. Neither liver, spleen, nor kidneys, contained infarcts. The vessels of the stomach and small intestine were distended. On removing the skull-cap the surface of both hemispheres was found to be covered with a thick layer of greenish pus. Thick pus coated the base of the brain involving the origins of all the cranial nerves. The cerebral vessels were examined for embolism without success. The substance of the brain was extremely soft, but there were no localized collections of pus. Sections of granulations on the tricuspid valve showed a few clumps of pneumococci. Sections of the liver showed a condition of extreme passive congestion with well-marked fatty change.

Bacteriological examination: A specimen of the cerebrospinal fluid was removed during the life of the patient. It contained large numbers of pus cells and pneumococci. At the post-mortem examination specimens of the heart-blood were taken before the heart was opened. Pneumococci were present in the films made from the heart-blood, and the pneumococcus was obtained in pure culture. Numerous pneumococci were found in films made from the pus obtained from the surface of the brain, and a pure culture of the pneumococcus was obtained from this source. The cultures of the pneumococcus were of typical appearance and killed inoculated mice in twenty-four hours.

The interesting features of the case are the age of the patient and the distribution of the lesions on the right side of the heart. The lesions were undoubtedly due to the pneumococcus, and at the post-mortem examination the lungs showed no evidence of broncho-pneumonia. Judging by the state of the ventricular wall and the appearance of the liver, it seems likely that valvular disease had been present for some considerable time. Indeed, chronic valvular disease may well have been present before the onset of the final infection. In any case it seems quite certain that the suppurative meningitis was secondary to the heart condition.

I am indebted to Dr. Firth for his kindness in allowing me to make use of the clinical notes of the case.

XLIV. STATISTICAL INVESTIGATION OF PLAGUE IN THE PUNJAB. SECOND REPORT: ON THE CONNECTION BETWEEN PROXIMITY TO RAILWAYS AND FREQUENCY OF EPIDEMICS.

BY M. GREENWOOD, JUNR.

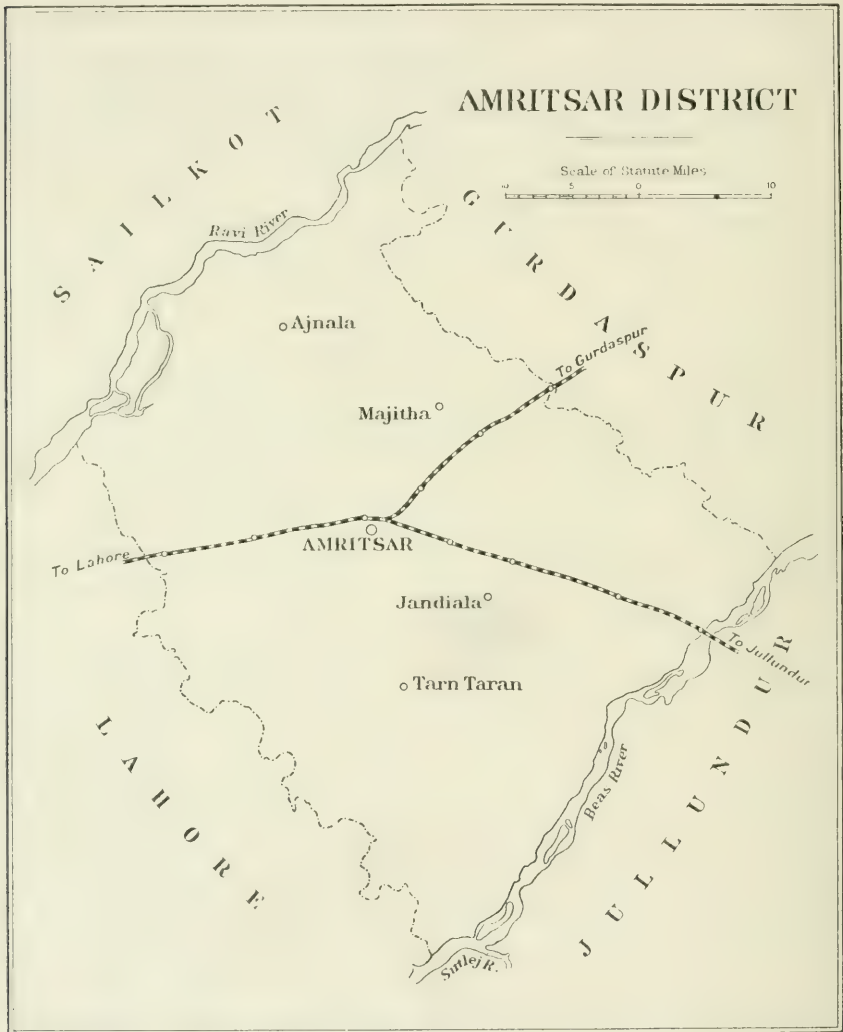
(*Statistician to the Lister Institute of Preventive Medicine.*)

With Map.

IN my former report (Vol. x. p. 424) the suggestion was made that proximity to means of communication might have an important influence on the distribution of plague epidemics within a district, and that some such disturbing factor might have been specially influential in the district of Amritsar, which is characterised by the extreme irregularity of its epidemic distribution. The present report is occupied with an analysis of material bearing on this subject.

The train of ideas which led up to the suggestion just noticed was briefly as follows. The evidence collected by Major Lamb, together with his and my analysis, tended to support the conclusion that an outbreak of plague in a village was more likely to be caused by the importation of the disease from outside, in the last resort from some relatively constant centre of infection, than by the rekindling of embers which had remained inactive since a former outbreak. But, if this view were correct, it should follow that villages in frequent communication with centres of almost constant infection, such as the large cities, ought to show a heavier rate of recurrence than villages of the same size but more secluded. The circumstances which make for free intercommunication fall into two classes. In the first place, the better a village is served by means of transit in the shape of roads and railways, the greater, other things being equal, will be the intercourse between its inhabitants and the outside world. A second class of influences cannot however be neglected. The racial peculiarities of the inhabitants, their religious and social customs and, in some cases perhaps, their occupations may make a considerable difference. It

might be that village A although badly served by railways or other means of transit is inhabited by a class of people who are peculiarly prone to migrate, while village B, in an ostensibly more favourable situa-



tion, contains a predominantly sessile population. I draw attention to these points because it is necessary to bear in mind that the complete solution of epidemiological problems such as we have to face in connection with plague demands a knowledge of many elements not at present in our

hands ; I am here concerned merely with a single one of the influences which are at work and, although the reader will perhaps concede it to be an important one, it is by no means the only nor even the prime factor. The point I have actually investigated is the connection between proximity to railways and the frequency of plague epidemics in the three tehsils of Amritsar district. I first marked on the map all villages which were situated not more than two miles from one of the three lines of railway, *i.e.* the lines from Amritsar to Lahore, Gurdaspur and Jullundur (see Map). The number so included was 155, but this figure is probably not accurate within, say, half a dozen either way, owing to the difficulty found in identifying the key numbers on the maps with the corresponding villages in the data lists, a difficulty in part due to the synonymy which is presumably an effect of transliteration.

The list obtained included two municipal towns of large size, Amritsar city and Jandiala¹, which are much larger than the other villages and possibly not comparable in other respects ; in the absence of specific mention to the contrary they are not taken account of in my analysis. The remaining villages are grouped in respect of plague history as shown in Tables I and II.

The first point to notice is that the distribution is unlike that of Amritsar district taken as a whole (Table III).

Had the latter distribution been valid for the selection we should have had the state of affairs indicated in Table III B.

TABLE I.

Summary of plague history of villages in Amritsar within two miles of the railway.

Number of infections	Number of villages
0	13
1	16
2	27
3	37
4	32
5	24
6	4
	153

¹ Re-measurement suggests that Jandiala is somewhat more than two miles from the railway.

TABLE II.

Annual incidence of plague in the selected villages.

Year of infection	Number of villages
Infected in 1st year	9
„ 2nd „	73
„ 3rd „	79
„ 4th „	114
„ 5th „	77
„ 6th „	97

TABLE III.

A. *General summary of the whole Amritsar district.*

Number of infections	Number of villages
0	155
1	183
2	211
3	230
4	169
5	93
6	21
	1062

B. *Numbers of villages within two miles of the railway actually infected compared with the expected numbers calculated on the basis of the whole district.*

Number of infections	Calculated number of villages	Actual number of villages
0	22.33	13
1	26.36	16
2	30.40	27
3	33.14	37
4	24.35	32
5	13.40	24
6	3.03	4
	153.01	153

It will be noticed that the selected villages exhibit a disproportionately large number of multiple infections and an unduly small number of unaffected and once affected members. What we have to determine is whether this difference is statistically significant or capable of explanation as a mere error of random sampling. The first question that arises is as to whether the difference is an effect of size variations, since we know that villages of large size are more likely to show multiple infections

than are small ones. Tables IV and V show the size distributions of the selection (inclusive of municipal towns) and of the whole district; Table V repeats the actual figures for the selection together with the calculated figures on the supposition that the selection is a random sample in respect of size. Testing in the usual way we find $P = .15$

TABLE IV.

*Population of villages within two miles of the railway
(inclusive of two municipal towns).*

Population	Number of villages	Population	Number of villages
0—300	24	3000—3300	1
300—600	46	3300—3600	0
600—900	25	3600—3900	1
900—1200	18	3900—4200	0
1200—1500	16.5	4200—4500	0
1500—1800	13.5	4500—4800	0
1800—2100	5	4800—5100	0
2100—2400	1	5100—5400	0
2400—2700	1	5400—up	3
2700—3000	0		155.0

TABLE V.

Populations of all villages in Amritsar District.

Population	Number of villages	Population	Number of village
0—300	248	3000—3300	7.5
300—600	299	3300—3600	5
600—900	194.5	3600—3900	4
900—1200	105.5	3900—4200	4
1200—1500	69.5	4200—4500	6
1500—1800	47.5	4500—4800	1
1800—2100	35	4800—5100	2
2100—2400	13	5100—5400	0
2400—2700	8	5400—up	6
2700—3000	6.5		1062.0

and, although the small groups at one end of the series tend to worsen the fit, it must be admitted that the selection contains an appreciably too small proportion of little villages, a fact which is favourable to a large number of multiple infections. In order therefore to decide whether the incidence of plague upon the villages within two miles of the railway is really excessive we must proceed further with our analysis.

Tables VII and VIII were next prepared and are self-explanatory. The reason for confining myself to villages not containing more than 2100 inhabitants was the small number and scattered distribution, in respect of size, which distinguish the larger villages. Table IX was then prepared; its method of construction will be plain from the following example.

TABLE VI.

Expected distribution of size in the selected villages on the basis of the returns for the whole district.

Population	Actual number	Calculated number
0— 300	24	36·20
300— 600	46	43·64
600— 900	25	28·39
900—1200	18	15·40
1200—1500	16·5	10·14
1500—1800	13·5	6·93
1800—2100	5	5·11
2100—2400	1	1·90
2400—2700	1	1·17
2700—3000	0	0·95
3000—3300	1	1·09
3300—3900	1	1·31
3900—4500	0	1·46
4500—5100	0	0·44
5100—etc.	3	0·88
	155·0	155·01

P (13 groups) = 0·15.

TABLE VII.

All Amritsar villages with populations up to 2100.

Population groups	Number of epidemics							Totals
	0	1	2	3	4	5	6	
0— 300	105	71	44	20	5	3	0	248
300— 600	43	75	81	70	23	7	0	299
600— 900	4	22	53	64	41·5	8	2	194·5
900—1200	2	10	15	29	31·5	16	2	105·5
1200—1500	0	1	11	18	25	11·5	3	69·5
1500—1800	0	1	5	7	15	18·5	1	47·5
1800—2100	1	1	2	13	7	10	1	35
Totals	155	181	211	221	148	74	9	999

TABLE VIII¹.

All selected villages with populations up to 2100.

Population groups	Number of epidemics							Totals
	0	1	2	3	4	5	6	
0—300	9	6	6	2	0	1	0	24
300—600	4	7	13	16	4	2	0	46
600—900	0	1	4	9	9	1	0	24
900—1200	0	1	3	5	6	3	0	18
1200—1500	0	0	1	2	7	4·5	2	16·5
1500—1800	0	1	0	0	4	8·5	0	13·5
1800—2100	0	0	0	1	0	3	1	5
Totals	13	16	27	35	30	23	3	147

Let us take the case of villages thrice infected. The chance of a village between 0–300 being three times affected is (Table VII) $\frac{20}{248}$, and therefore in 24 villages (Table VIII) we should expect $\frac{20 \times 24}{248}$ instances. Similarly the contribution of the villages between 300 and 600 should be $\frac{70}{299} \times 46$. The total number should be

$$\frac{20 \times 24}{248} + \frac{70 \times 46}{299} + \frac{64 \times 24}{194 \cdot 5} + \frac{29 \times 18}{105 \cdot 5} + \frac{18 \times 16 \cdot 5}{69 \cdot 5} + \frac{7 \times 13 \cdot 5}{47 \cdot 5} + \frac{13 \times 5}{35} = 33 \cdot 7.$$

Testing for goodness of fit, one finds $P=0 \cdot 10$. The agreement is not close as it is, but the test is really too favourable to the chance of agreement for the following reasons. The totals (Table VII) upon which the prediction is based include the villages within two miles of the railway. But these latter actually form 14·7 per cent. of all the villages, hence some degree of approximation to the calculated values was only to be expected. A fairer test of differentiation seems to be to compare the actual distribution with that calculated on the basis of the villages which were more than two miles from a railway. For this purpose, Table X was prepared by subtracting the entries in Table VIII from the corresponding values in Table VII. From this table, the results given in Table XI were obtained by the process just illustrated. The value of P for this table shows that the agreement is considerably worse, that the selection exhibits too high a proportion of villages frequently infected and too low a proportion of villages rarely plague-stricken. All these results point to the conclusion that villages within reach of a railway are not a random sample of the whole of the villages. One further test may be mentioned.

¹ After the analysis had been completed another village was satisfactorily identified and should have figured in the selection. As no appreciable difference would be made in the results the tables have not been re-worked.

TABLE IX.

Number of epidemics calculated for the selected villages on the basis of the whole district, attention being paid to size. For villages with populations up to 2100.

Number of epidemics	Actual number of villages	Calculated number
0	13	17.75
1	16	23.49
2	27	30.14
3	35	33.67
4	30	25.71
5	23	14.50
6	3	1.73
	147	147.00

$$P=0.10,$$

TABLE X.

Number of epidemics in villages more than two miles from the railway with populations up to 2100.

Population groups	Number of epidemics							Totals
	0	1	2	3	4	5	6	
0—300	96	65	38	18	5	2	0	224
300—600	39	68	68	54	19	5	0	253
600—900	4	21	49	55	32.5	7	2	170.5
900—1200	2	9	12	24	25.5	13	2	87.5
1200—1500	0	1	10	16	18	7	1	53
1500—1800	0	0	5	7	11	10	1	34
1800—2100	1	1	2	12	7	7	0	30
Totals	142	165	184	186	118	51	6	852

Let there be two samples n_1, n_2 : let $n_1 = p_1 + q_1$ where p_1 = number marked by some character (*e.g.* the fact of having three epidemics of plague); let $n_2 = p_2 + q_2$. The standard deviation of $p_1 = \sqrt{\frac{p_1 q_1}{n_1}}$ and of $p_2 = \sqrt{\frac{p_2 q_2}{n_2}}$. The percentage difference between the results of the two samples is $100 \left(\frac{p_1}{n_1} - \frac{p_2}{n_2} \right)$ and the probable error of the difference is:

$$67.449 \times \sqrt{\frac{p_1 q_1}{n_1^3} + \frac{p_2 q_2}{n_2^3}} \dots \dots \dots (1).$$

TABLE XI.

Number of epidemics calculated for the selected villages with populations up to 2100, on the basis of the rest of the district, attention being paid to size.

Number of epidemics	Actual number of villages	Calculated number
0	13	18·52
1	16	24·61
2	27	31·23
3	35	34·19
4	30	24·95
5	23	12·10
6	3	1·40
	147	147

$$P=0\cdot006.$$

We might compare the differences of percentages of villages infected 0, 1, 2, etc. times in each population group from the two sets of villages with the probable errors determined by (1).

This test is open to criticism. The use of the probable error based on the standard deviation as determined from the ordinary binomial requires for complete validity two assumptions, (1) that the events are really independent, (2) that the chances for and against the event are not very widely different. With regard to the latter objection, it can be met by only comparing groups in which not less than, say, 10% of the villages were infected. The former objection cannot, however, be evaded in this way. It is likely that the fact of one village being infected is not independent of the presence of infection in another village. It must, however, be observed that if plague outbreaks are mainly attributable to recrudescence and not to importation, the condition would be more nearly fulfilled, although even in that event we cannot neglect entirely the factor of common meteorological or biological conditions. On the whole, it seemed just worth while applying the test to a few groups; this has been done and the results appear in the short Table XII. Although the differences are all less than three times their probable errors, they all exceed two and a half times this function so that it may justly be said the inferences to be drawn in no way conflict with those already stated.

Having reached the conclusion that proximity to a railway does increase the liability of a village to recurrent epidemics, I attempted to measure the intensity of the relationship. To do so we must employ

the methods of multiple correlation, for we have not two variables but three, viz. (1) proximity to a railway, (2) size, (3) number of epidemics. We must determine the correlation between two of these variables for a constant value of the third, the coefficient of "partial" or "net" correlation¹.

TABLE XII.

Comparison of certain groups of villages.

Group		Percentage of such villages in the sample within 2 miles of the railway	Percentage of such villages among the villages more than 2 miles from the railway.	Difference $\pm$ probable error	Difference divided by its probable error
Population	Epidemics				
0—300	1	15.22	26.88	-11.66 ± 4.04	2.89
300—600	3	34.78	21.34	$+13.44 \pm 5.05$	2.66
600—900	4	37.50	19.06	$+18.44 \pm 6.97$	2.65

I first determined the ordinary coefficients of correlation and found:

- (1) Between size of village and number of epidemics, $r = .594$.
- (2) Between proximity to railway and size of village, $r = .154$.
- (3) Between proximity to railways and number of epidemics, $r = .265$.

(1) was determined by the ordinary product moment method, (2) by a new process due to Pearson², (3) from a fourfold table³, the divisions being for villages (*a*) those within two miles of the railway, (*b*) those more than two miles from the railway; for epidemics—(*a*) not more than two epidemics inclusive, (*b*) three or more epidemics. Finally I obtained the correlation between proximity to railways and number of epidemics for a constant size of village. This was found to be .22. The question then arises as to the probable error of this coefficient. Were the partial correlation deduced from coefficients evaluated by the product moment method, it is known⁴ that the ordinary formula can be used⁵. In this case, one at least of the total correlations is subject to a larger probable error than that based on product moments. I think, therefore, we should assume that the

¹ Yule, *Proc. Roy. Soc.* 1897, LX, 477.

² Pearson, *Biometrika*, 1909, VII, 96.

³ Pearson, *Phil. Trans. A*, 1895, CXCIV, 1.

⁴ Yule, *Proc. Roy. Soc. A*, 1907, LXXIX, 182. Heron, *Biometrika*, 1910, VII, 411.

⁵ $0.67449 \times \frac{1-r^2}{\sqrt{n}}$.

partial coefficient is subject to a probable error about three times the size of that given by the ordinary formula¹. The formula gives in our case .02, so that we can reasonably take the correlation between proximity to railways and number of epidemics as $.22 \pm .06$. As the coefficient is more than three times its probable error, we may have some confidence that it indicates significant relationship between proximity to railways and number of villages.

One further point may be considered. Can we explain, on the basis of this work, the heterogeneous character of the epidemic distribution in Amritsar noticed in the first report? We can, I think, safely assert that this is one of the factors in operation, but certainly not the only one. I find on considering the villages exclusive of these within two miles of a railway, that the distribution is by no means a random one. This was to be expected, since even in regard to proximity to means of communication, the analysis here described is not exhaustive. Nearness to highways for wheeled traffic is very likely also a circumstance of importance, but, since we have evidently no satisfactory way of judging as to which roads marked in the maps are or are not greatly frequented, statistical analysis can hardly be applied to the subject.

The conclusion to be drawn from this study is therefore, that *the villages near a railway are subject to a higher rate of plague incidence than other villages and that this difference is not to be attributed to the smallness of our experience but may be considered an epidemiological fact.*

We must now inquire more particularly what is the epidemiological significance of the results obtained. Proximity to a line of railway might lead to an increased incidence of plague for all or any of the following reasons.

(1) Increased *personal* communication between villagers and centres of constant infection.

(2) Increased facility for the transit of merchandise, especially grain which might convey infected rats or possibly fleas.

(3) Some peculiarity either of the villages or the inhabitants of villages lying near the railway unconnected with (1) and (2). Since we have no extrinsic evidence pointing to condition (3), we are, for practical purposes, left to decide between (1) and (2). In a district containing large and important cities, cities of more than local fame, personal intercourse by railway is likely to be maximal, especially if any section of the

¹ This is a very generous allowance; in the particular case, I find, the probable error of the total coefficient (3) only 2.2 times the value deduced by the ordinary method.

inhabitants owing to racial or religious peculiarities are given to travel. On the other hand in a district without large towns personal intercommunication between villages and such towns as exist will be much less, although the railway may be extensively used for the transit of goods. Amritsar fulfils the conditions requisite for much personal intercourse. Amritsar city is one of the largest and most important, if not the most important, cities in the Punjab. The district contains a considerable percentage of Sikhs who, as I understand, are extremely prone to travel and regard Amritsar city with religious veneration. On the other hand, Rohtak is an almost purely agricultural district. It contains no towns of more than local importance and practically no Sikh inhabitants. These remarks apply in the main to a third district, Gujrat, the data from which came into my hands since the results last communicated were worked out. From the information before me, I should infer that the manufacturing or commercial interests may be stronger in Gujrat than Rohtak but that from the point of view of personal intercourse it falls into line with that district. If, therefore, (1) be the true explanation, we ought to find that the partial correlation coefficient (constant population) is larger in the case of the data for Amritsar than for Rohtak or Gujrat.

Taking the same limits of size and adopting the same measure of accuracy as for Amritsar, I calculated the various constants for Rohtak and Gujrat, finally obtaining as partial coefficients of correlation between proximity to railways and frequency of epidemics, for a constant population :

Rohtak, $\cdot 12 \pm \cdot 09$,

Gujrat, $-\cdot 18 \pm \cdot 06$.

I have adopted the same generous margin, viz. three times the probable error given by the ordinary formula; allowing for this, we can hardly say that there is much evidence of any influence in the case of Rohtak. The coefficient for Gujrat is actually negative, pointing to some other influence, possibly a spread of infection from the margin of the district which obliterates any effect such as we have found in Amritsar.

These results amount, I think, almost to a proof of the correctness of hypothesis (1).

In our previous reports, Major Lamb and I devoted much attention to ascertaining whether the genesis of plague in the districts we investigated were in the main an affair of importation of infected

material (or animals) or due to the recrudescence of a dormant process. As stated at the beginning of this report, were the former hypothesis to express a truth, we should expect villages near an important means of communication to suffer with excessive frequency from epidemics of plague. If on the other hand, an epidemic is generally due to recrudescence, it is difficult to see why proximity to a railway should be of any importance unless we adopt (3) *supra*, which, as previously remarked, has nothing in its favour.

But not only do we conclude generally in favour of the importation hypothesis in the case of Amritsar, further we have evidence as to the conditions under which it will be valid. A comparison of the three districts seems to make it highly probable that it is importation by persons or personal effects which determines the spread of epidemics, not so much the mercantile goods' traffic.

While fully admitting the imperfections of our material from the statistical point of view, I think the conclusions just stated must be regarded as at least highly probable. It is, however, quite clear that the factor here demonstrated is not the only nor perhaps the chief one at work. This is shown by the absolutely small value of the coefficient even for Amritsar district, where the conditions are most favourable, and its total extinction in the agricultural districts of Rohtak and Gujrat.

CONCLUSIONS.

1. *In districts containing large cities, villages near a line of railway are liable to a higher rate of plague epidemics than villages not so situated.*

2. *This is probably due to increased opportunities for personal transit, not to the exportation or importation of merchandise.*

3. *In a district favourably situated for personal intercommunication, the spread of plague can be much better explained on the hypothesis of reimportation than on that of recrudescence.*

4. *In purely or mainly agricultural districts, proximity to railways does not increase the liability to plague outbreaks.*

APPENDIX.

Further note on the actual and calculated frequencies of recurrent epidemics of plague.

In my former report (Vol. x. pp. 425-7 and Table XXIX) a special comparison was instituted between the actual frequencies of villages affected in successive years and the numbers calculated on the assumption that plague in one year did not predispose to an epidemic in the same village in the following year. In testing agreement between expectation and observation, I adopted the process used in earlier sections of the report. Now, while the value of χ^2 is evidently some measure of agreement in all cases, the value of P given by such a table as XXIX (*op. cit.*) cannot be compared strictly with the results obtained from the other tables since the whole of the chance distribution was not used. As the test presupposes a complete distribution, I have extended the comparison to the whole of the possible categories and we obtain the accompanying Table XIII. In this table the figures enclosed in brackets are the calculated frequencies on the hypothesis of independent chances. We shall obtain the same total frequency for calculated and observed values by grouping the results in several ways.

(1) If we consider each vertical column of the table as a distribution, we find the agreement to be measured by the values of P entered at the foot of each column.

(2) If we consider each sequence of two years separately and take a separate subdivision for each group of villages, giving 20 subdivisions in all, we have :

1903-4, $P=0.86$; 1904-5, $P=0.99$; 1905-6, $P=0.70$; 1906-7,
 $P=0.49$.

(3) If we group all the villages together, irrespective of size, for each sequence, leaving only four groups, the values of P become 0.02, 0.78, 0.04, 0.39.

Of these arrangements (1) is perhaps to be preferred.

As will be seen from the general trend of the values, this more detailed comparison leads to precisely the same conclusion as that given in the text. In other words, the difference between observed and expected values in this series is not sufficient to warrant a belief that recurrences occur with undue frequency. The conclusions based upon this finding in the first report may therefore stand.

TABLE XIII.

Recurrence of plague in villages: Amritsar district.

The figures are numbers of villages, those in brackets being the numbers calculated on the supposition that the distribution of plague in one year is independent of the preceding and succeeding epidemic distribution.

Year	Population groups				
	400-500	500-700	800-1000	1000-1200	1200-1400
1903 not 1904	32 (34·35)	36 (44·73)	29 (32·07)	16 (17·97)	9 (10·35)
1904 not 1903	12 (14·35)	28 (36·73)	14 (17·07)	12 (13·97)	7 (8·35)
1903 & 1904	17 (14·65)	39 (30·27)	32 (28·93)	27 (25·03)	25 (23·65)
Neither year	36 (33·65)	63 (54·27)	22 (18·93)	12 (10·03)	5 (3·65)
1904 not 1905	14 (12·56)	22 (24·62)	4 (6·64)	6 (8·15)	5 (4·17)
1905 not 1904	40 (38·56)	60 (62·62)	41 (43·64)	20 (22·15)	13 (12·17)
1904 & 1905	15 (16·44)	45 (42·38)	42 (39·36)	33 (30·85)	27 (27·83)
Neither year	28 (29·44)	39 (36·38)	10 (7·36)	8 (5·85)	1 (1·83)
1905 not 1906	38 (39·12)	85 (87·29)	52 (55·62)	25 (28·48)	23 (25·22)
1906 not 1905	11 (12·12)	8 (10·29)	1 (4·62)	3 (6·48)	0 (2·22)
1905 & 1906	17 (15·88)	20 (17·71)	31 (27·38)	28 (24·52)	17 (14·78)
Neither year	31 (29·88)	53 (50·71)	13 (9·38)	11 (7·52)	6 (3·78)
1906 not 1907	15 (13·86)	8 (15·01)	4 (8·58)	8 (8·79)	7 (4·07)
1907 not 1906	36 (34·86)	57 (64·01)	43 (47·58)	25 (25·79)	25 (22·07)
1906 & 1907	13 (14·14)	20 (12·99)	28 (23·42)	23 (22·21)	10 (12·93)
Neither year	33 (34·14)	81 (73·99)	22 (17·42)	11 (10·21)	4 (6·93)
	$P=0·99$	$P=0·27$	$P=0·53$	$P=0·95$	$P=0·80$

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XLV. STATISTICAL INVESTIGATION OF PLAGUE IN THE PUNJAB. THIRD REPORT: ON SOME OF THE FACTORS WHICH INFLUENCE THE PREVALENCE OF PLAGUE.

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With Map and 5 Figures.

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I. *Introduction.*

IN a first report, written in conjunction with Major Lamb¹, evidence was presented which made it seem probable that the recurrence of plague in the villages of the Punjab and United Provinces, which had been specially investigated, could in the main be attributed to reimportation of the disease; such a mode of origin appeared epidemiologically of greater significance than a possible awakening of

¹ "On the spread of epidemic plague through districts with scattered villages." *Journ. Hyg.* x. (1910), p. 350.

some *materies morbi* which had remained dormant since an earlier visitation. We also pointed out that the proportion of small villages which remained free from plague in any epidemic was much higher than that of large villages or towns (see especially Tables LIV–LIX, pp. 659–661, and i. p. 676). It was further shown that, apart from questions of size, the distribution of infected villages, particularly in the Amritsar district, suggested that certain regions might be peculiarly exposed to infection. The suggestion was made that proximity to habitual lines of travel was a factor in this result, railways being specially mentioned. In a second report (p. 47 *supra*) I have shown that, in Amritsar district, proximity to the railway has undoubtedly influenced the distribution of infected villages. At the same time, however, it was pointed out that the result of a similar inquiry in the case of another district, Gujrat, indicated that proximity to railways could not be the sole nor even the chief factor in disseminating plague.

In the two previous reports, attention was almost entirely confined to the simple fact of infection or non-infection, a division into two mutually exclusive classes being all that was attempted. Little or no heed was paid to the remarkable differences in rates of mortality among infected villages, which are nevertheless sufficiently marked in character. In order to give the reader of this report some idea of such differences, Tables I–VIII have been prepared; they are mere random samples intended as illustrations of a host of similar facts. These and kindred observations led me to formulate an epidemiological problem in the following terms—What circumstances determine the *extent* of an epidemic when plague has once manifested itself, why is it that such great discrepancies are observable in the rates of mortality? The present report is a systematic attempt to analyse the more prominent of the factors which could be thought responsible for the results. While it will undoubtedly be found that much remains obscure, I am hopeful that some light has been thrown upon points not without epidemiological importance.

II. *Nature of the statistical materials and possible sources of error and uncertainty.*

The districts under observation are Gujrat, Hoshiapur, Rohtak and Amritsar in the Punjab, and Mozuffarnagar in the United Provinces. A few general particulars relating to these districts have been collected in Tables IX and X. It will be noticed that Amritsar is the only one

in which agriculture does not employ at least half the inhabitants and also the only one possessing a considerable urban population. The returns of plague-deaths from the numerous villages within these districts were made under the personal supervision of the late Major Lamb and numerous corrections on the data sheets together with the correspondence exchanged between Major Lamb and the local officials testify to the care with which the figures have been scrutinised. As has been frequently pointed out, however, some degree of uncertainty must always exist. Thus, when the prevalence of plague is low, there is perhaps a tendency to report deaths due to plague under other headings; conversely when the disease is widespread, deaths not caused by plague may possibly be credited to this disease. Naturally the magnitude of this error cannot be determined from internal evidence¹, comparable statistics of deaths from all causes are not at hand, but its possible existence must not be ignored. A second source of error is the fact that the population returns are based on the census of 1901, *i.e.* they refer to a date several years earlier than most of the epidemics in our series. If we could be certain that all the villages have increased or decreased in the same ratio, percentages calculated on the 1901 figures while absolutely wrong would still be comparable *inter se*. Such an assumption would, however, require strong external evidence to justify it and we may be fairly confident that the (compulsory) adoption of a 1901 population basis has introduced a somewhat considerable source of error into our discussion of mortality rates. A third source of error, or rather, to speak more correctly, of uncertainty, is due to the impossibility of checking the returns of deaths in our data schedules against those which figure in the weekly lists issued by the Government of India. The summed totals from the Commission's data are widely at variance with those given in these weekly lists. It would seem, however, that the difference is not caused by any serious error in either set of figures, since there is a tolerably close agreement between both records with regard to specific towns. The most plausible suggestion is that there has been some different delimitation of the districts for the purposes of the two series. One remark may be made with regard to the weekly returns; little or no weight can be attached to case mortality deduced from a comparison of the reported seizures with the reported deaths for the same or any previous week. In many

¹ "From a careful study of deaths from all causes contained in the death registers we have arrived at the conclusion that very few deaths from plague escape notification." ("Plague in Belgium," *Journ. of Hyg.* x. 1910, p. 455.)

districts such a comparison would lead (has indeed led certain newspaper writers) to the conclusion that the average case mortality is well over 90 %, an improbable figure for bubonic plague.

It will hardly be questioned that the material upon which my analysis depends is subject to very considerable errors. These errors reflect in no way upon those who have spent so much time and care upon collecting and revising the data; indeed it may safely be asserted that the present material has attained a higher standard of precision than any previous records at all commensurate with it in scope. Nevertheless, the possible sources of error are so important that statistical deductions can only be made with considerable hesitation. When the whole or most of the evidence points consistently in any one direction, we may justly have some confidence that the conclusion indicated is sound; but we must neither push our inferences too far, nor expect very detailed results. It is for these reasons that I have often presented the same material tabulated with what may seem unnecessary repetition and that I have drawn conclusions rather cautiously, even when the results of tabulation or analysis seem to force them upon one's notice. I have also in most cases omitted such subdivisions of data as would fairly involve the consideration of the so-called "errors of random sampling." I shall, indeed, from time to time refer to certain statistical questions of this nature, but in most cases such applications would only tend to clothe the results in a garment of spurious precision. In this connection I may say that tabulation has often been carried to decimal places beyond the limits of material accuracy. This has been done partly in the interests of neat statement, but more to avoid the illogical but almost instinctive conclusion on the reader's part that results given in round numbers are based on careless or approximate methods of calculation. I have thought it well to emphasize this question of "material errors" at the outset; if the subject is not again discussed in detail, I hope the reader will not imagine it to have been lost sight of and that, on the other hand, doubt or hesitation will not be attributed to pedantry. I have used my best efforts to place just as much weight upon my statistical foundations as they will bear and no more.

III. *The influence of length of exposure to infection upon total mortality.*

It has already been stated that previous work inclines one to believe that the origin of most village outbreaks is the importation from elsewhere of the disease—how this takes place will be matter for subsequent consideration. It will at once seem probable that a village which has become infected early in the season must show a greater number of cases in the whole epidemic than one not “exposed to risk” until later in the plague season, which extends in these districts roughly from November to July. Hence it might be supposed that the differences in rate of mortality as exhibited in the annual figures, which were the starting point of the present inquiry, depended merely upon this circumstance. Tables XI–XVIII contain a first study of the influence of length of exposure to infection upon total mortality; they were prepared in the following way.

All the Hoshiapur villages without exception, and all villages of less than 10,000 inhabitants in the four other districts, were grouped according to the month of first infection. That is to say, a list was made of villages which first reported cases in—*e.g.*—March, their total population, total numbers of deaths, percentage mortality, etc. were noted and the same process was applied to every other month in the plague season. The very few¹ villages which reported cases as early as October or November were grouped with December infections and the still fewer villages which escaped until August either omitted or reckoned in the July total. If a village had a free interval of three months or more—*e.g.* if it returned cases in January and no more until May—it was reckoned twice, once under each month. The salient features of these tables are indicated in the following notes.

Amritsar. 1902–3. The February infections show a higher average mortality than those of January.

1903–4. A steady decline from February. The first two months are based upon statistically inadequate returns.

1904–5. A similar picture to that of 1903.

1905–6. A practically uniform decline; there were only two infections before January.

¹ For the epidemics and districts where these conditions fail a separate table (XVIII A) has been prepared.

1906-7. Villages first infected in January, February or March differ little in rates of total mortality or average populations.

Hoshiapur. 1901-2. February and January rates similar but the mean population of the January villages markedly higher.

1903-4. February rates markedly higher than those of previous or subsequent months. (Let me repeat that what I call *e.g.* the February rate means the total rate *for the whole epidemic* of villages first infected in February, *not* the number of deaths reported in that month alone.)

1904-5. No great diminution in the rate before May, but villages first infected in December show the greatest rate.

1906-7. The marked fall does not begin until April. February worse than January.

Gujrat. 1903-4. Very irregular. The first three months based on too small figures.

1904-5. A fairly regular fall from the beginning but the March and April rates are sensibly equal.

1906-7. February once again predominant, but the earlier months depend on small figures.

Mozaffarnagar. 1903-4. Figures too small to be reliable.

1904-5. Shows a tolerably smooth fall.

1905-6. Also unreliable numbers.

1906-7. A maximum in March.

Rohtak. 1904-5. December below any month before April.

1906-7. February and March about equal. December and January unreliable.

The general conclusion suggested by a study of these tables would be that, on the whole, the earlier the infection is introduced the greater the resultant mortality but that the relation is neither very close nor straightforward. Let us now scrutinise the figures more closely. As will have been noticed in the tables, the average population of villages infected early in the season is generally much greater than that of villages having a longer free interval (a circumstance which might have been predicted from the evidence contained in earlier reports). It is therefore necessary to isolate the influence of earliness of infection upon total mortality from that of size; this has been attempted in two ways. Before discussing these methods, however, the reader must be clear as to exactly what problem we are now considering.

We are at present solely occupied with the influence of length of exposure upon total mortality. The plague season extends for all practical purposes when we deal with villages, not large towns, from

November to July; hence a village reporting cases in December has before it eight months during which cases might occur; if the infection does not reach the village until May then only three months can yield cases. It does not, of course, follow that these possibilities are realised, the force of the infection may spend itself in one or two months; so to speak, the danger is potential and need not become actual; this distinction will be examined later on. If now we take the number of months from the date of first infection to the end of the season as one variable and the total recorded deaths as the other variable, the correlation between the two will be a measure of the relationship between total mortality and length of possible exposure. If for number of deaths we substitute population, the correlation will measure the relation between size of village and length of exposure. It must be carefully noted that the former of these correlations does not measure the relation between any meteorological condition and the number of plague deaths. Such influences require and will receive separate consideration, but they cannot profitably be discussed until the factor of mere duration in time has been examined and measured. I now return to the methods which have been employed to study this problem.

(1) Groups of villages having nearly the same population were formed and the correlation between number of months of exposure and total deaths was determined by the direct product moment method without grouping. By this process the population factor was experimentally kept constant, but only very roughly, as will be seen.

(2) All villages of not more than 3000 inhabitants were tabulated, together with their plague histories, and the following coefficients evaluated, by the product moment method.

- (i) Absolute population and number of cases.
- (ii) Absolute population and length of possible exposure.
- (iii) Length of possible exposure and total number of deaths from plague.

We have, if r_{pd} = correlation between absolute population and length of exposure, r_{pm} = the correlation between absolute population and absolute number of deaths and r_{dm} = the correlation between absolute number of deaths and length of possible exposure,

$$\frac{r_{dm} - r_{pd} \times r_{pm}}{\sqrt{1 - r_{pd}^2} \times \sqrt{1 - r_{pm}^2}}$$

for the correlation between length of possible exposure and number of deaths for a constant population.

The results of both methods appear in Tables XIX–XXI¹. Scientifically the second process is to be preferred because it was impossible to obtain a reasonably large group of villages affected in any one epidemic which were sensibly equal in populations. It is not, therefore, surprising that the coefficients in Table XIX are very variable although of the same order of magnitude as those in Table XXI. With special reference to the values of Table XXI it will be noticed that all epidemics in Hoshiapur give results in good agreement one with the other. One Gujrat coefficient is large, one Amritsar and one Mozuffarnagar value seem unduly low; in the latter case the arithmetical work has been repeated without the discovery of any slip in computation.

The question then arose whether the relatively small sizes of the coefficients were due to any marked departure from linearity so that the coefficient ceased to be a satisfactory measure of inter-relationship when taken by itself. It is hardly possible, in the present state of knowledge, to consider the partial regression coefficients directly from this point of view; sufficient information for our purpose can, however, I think, be obtained from a study of the total regression of deaths upon length of exposure. The mean numbers of deaths for each length of exposure were plotted from each set of data. The resulting points were not collinear but it was noticed that the most divergent means were based on few observations. In the case of Hoshiapur 1906–7 the nature of the regression was completely studied. In this case r was $\cdot 281$, η (the correlation ratio²) $\cdot 287$ and the ratio of $\eta^2 - r^2$ to its probable error³ $1\cdot 34$. In other words the regression was effectively linear. The diagram (Fig. 1) shows the mean points and the best straight line; it should be noted that in fitting the line each point is weighted with the number of observations on which it is based, so that the diagram exaggerates the divergence from linearity. I also found that the omission of the scattered observations derived from the early months of the epidemic did not, in the cases tested, lead to very substantial alterations in the coefficients although their values were increased. It does not, therefore, seem probable that a close relationship between length of possible exposure and total mortality has been masked by some peculiarity in the regression curve.

¹ Space does not permit of publishing all the correlation tables; as an illustration, the set relating to Amritsar 1902–3 is given (Tables XXI A, XXI B, XXI C).

² Pearson, *On the General Theory of Skew Correlation and Non-Linear Regression*, London, 1905.

³ Blakeman, "On Tests for Linearity of Regression in Frequency Distributions," *Biometrika*, iv. (1905), p. 332.

I next considered the relation between possible and actual exposure, *i.e.* I ascertained whether those villages which, owing to early importation of disease, might have been infected in the greatest number of months actually were so. Table XXII shows the average durations of plague in villages first infected in each month in sequence, for two epidemics which attacked a sufficiently large number of villages to render mean values significant. Naturally the actual is less than the possible exposure but varies fairly uniformly with the latter (Fig. 2). I next attempted to measure the influence of date of first infection upon total mortality, *i.e.* I sought to discover if more cases resulted from infection *n—e.g.*—months 1 and 2 than from infection in months 2 and 3. In

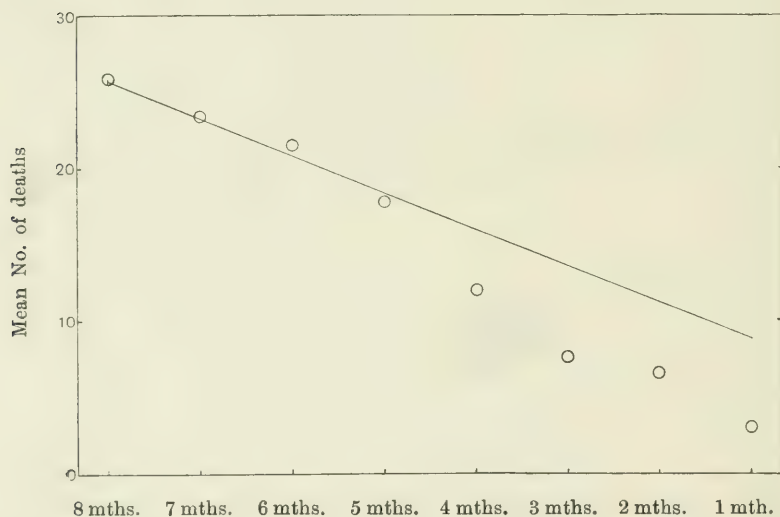


Fig. 1. Hoshiarpur, 1907. Mean no. of deaths for villages exposed during different periods. (The line is $y = .24x$ where y is measured from the mean no. of deaths, 19.1, and x from 5.25 months. The units are 10 deaths and 1 month.)

Gujrat for the epidemic of 1906-7 (villages of populations up to 3000) villages which reported deaths in two months only were tabulated, the three coefficients of correlation were calculated and the value of the relation between date of first exposure and number of deaths for a constant population proved to be $.26 \pm .04$. It would therefore seem that given an equally long effective exposure there is a slight tendency for more deaths to occur early in the season than later on. All these

results tend very clearly to show that two factors influence the death rate, (a) length of possible and actual exposure, (b) *date* of first exposure. They show not less clearly that neither of these factors nor both combined suffice to explain the wide discrepancies in death rates which were the starting point of the inquiry.

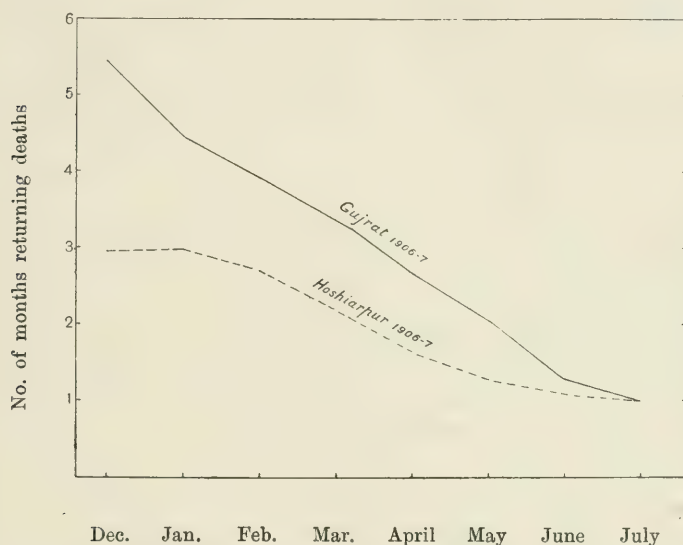


Fig. 2. Month of first infection.

The conclusions of this section may be summarised in the following way and, in view of the reasonable uniformity in analytical results, I have fair confidence that they correctly represent the epidemiological facts.

(1) *Other things being equal*, the rate of mortality varies with the length of possible and actual exposure but the relation is not close.

(2) Given the same period of actual exposure, more deaths will result from infections early in the epidemic season.

(3) Large villages tend to be infected early in the season, but the relation between size and date of infection is not close.

(4) The bulk of the difference between rates of mortality in individual villages of the same size cannot be explained completely by these results.

IV. *The relation between absolute size and relative mortality in villages.*

In Tables XXIII and XXIV, the data are so presented as to allow of a comparison between the rates of plague mortality in large and small villages. Table XXV gives the urban data while Tables XXVI—XXXII permit of a more detailed analysis of the village returns.

The general trend of these figures points to the conclusion that small villages are usually subject to a heavier rate of mortality, *when infected*, than larger ones. In certain cases, especially perhaps Gujrat, the difference is very considerable; in one instance, Rohtak, the rates go in the opposite direction. As will appear, this exception can be partly referred to the local differences which are found in Rohtak in respect of incidence, but even making this allowance, the Rohtak returns must be considered to form an exception from the present standpoint (Table XXXII)¹.

Before we attempt to explain these differences it may be asked whether they can possibly be referred to errors of random sampling and a few words may be devoted to this point.

If we wish to determine the probable errors of mortality rates for plague the usual method, founded on the binomial standard deviation, is inapplicable since the condition that the events are independent one of another is not fulfilled². The following modification of the usual process is perhaps worthy of adoption in such instances. We will take a specific example.

In Gujrat, during 1906–7, considering only villages which first reported deaths in March, the following results were obtained:

Villages of Populations 1000—2000.

Total population	Total deaths	Death-rate per cent.
36461	3983	10·92

Villages of Populations less than 700.

38522	5779	15·0
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Let us calculate the error of sampling in this case.

The weekly official returns enable us to determine the number of deaths in each month throughout the epidemic, but since the total

¹ See also Tables LXV and LXVI.

² See Yule, "An Introduction to the Theory of Statistics," London, 1911, Chap. XIV; Greenwood and Candy, *Journ. Roy. Statistic. Soc.* LXXIV, 1911, pp. 375–6; Yule, *ibid.* 398–9.

deaths recorded in the data prepared for Major Lamb do not correspond exactly with the weekly figures (*vide supra*) the latter were reduced to the same dimensions as the former, by multiplying throughout by

$$\frac{\text{Total Deaths in Commission's Data}}{\text{Total Deaths in the Weekly Returns}}$$

In the end, the following table was obtained :

Month	Population at risk	Chance of a plague death. Assuming independence of chances
March	234,017	·0221
April	413,096	·0247
May	505,874	·0436
June	509,820	·0199
July	503,481	·0029

We might consider each month's "exposed to risk" as a separate "population" and the chances of each person dying of plague as independent and constant *for that month*. Then, if the most probable number of deaths in any month be m , and the binomial standard deviation for the same month σ_m , the total number of deaths to be expected throughout the season will be $S.m$ with a standard deviation of $\sqrt{S\sigma_m^2}$. The calculation was carried out as shown in the next table :

A. *Villages of Population 1000—2000.*

Month	Expected deaths	Population at risk	Square of standard deviation
March	804	36,461	786·26
April	882	35,657	860·18
May	1517	34,775	1450·82
June	662	33,258	648·83
July	96	32,596	95·72
August	—	32,500	—
		3961	3841·81

S.D. 61·98. Prob. error 41·8

B. *Villages of Population 0—700.*

March	850	38,522	831·25
April	932	37,672	908·94
May	1603	36,740	1533·07
June	699	35,137	685·09
July	101	34,438	100·70
August	—	34,337	—
		4185	4059·05

S.D. 63·71. Prob. error 42·97

Finally, we reach :

Population-group	Calculated number of deaths	Actually observed number of deaths	Difference between the actual and calculated numbers divided by the latter's "probable error"
1000—2000	3961	3983	·53
0—700	4185	5779	37·1

If such a method of testing be admitted (it is at best a rough and ready process to be used with caution) the difference between the rates of mortality in the example can hardly be due to errors of random sampling.

The question of how to calculate probable errors in material of this class has a certain theoretical importance; practically, in the case before us, I do not attach any great value to the inquiry. We are only interested in large differences, and it may be doubted whether errors of random sampling are of much importance in comparison with the material errors of record mentioned above (p. 64). The numbers are large and the reader should examine the evidence as a whole, particularly Tables XXIII—XXXII. He will then, I believe, reach the conclusion that, on the average, in most epidemics the relative mortality from plague in small villages actually exposed is greater than upon large ones, the conclusion stated a few pages back. It must be remarked again that the proportion of villages which *escape altogether* is larger when we deal with the smaller communities (see Tables XXXIII, XXXIII A, and also this *Journal*, Vol. x. pp. 351, etc.). Table XXXIV illustrates the difference in the percentage rates which is effected by the inclusion not only of villages actually infected but also of those which might have been¹. It will be noticed that in some, but not all, cases this inclusion has sufficed to turn the scale in the other direction. On the whole, then, a review of the somewhat considerable mass of data here collected seems to prove that, given an equal period of exposure to infection, small villages suffer more severely from plague than large ones, and we have no ground for attributing this result to errors of sampling. Can they be due to errors of record? Evidently a mistake in the returns from a small village will lead to a relatively greater error in the mortality rate than an inaccuracy of the same absolute magnitude in a village of larger size. But it would be futile to seek an explanation of our result in this way. We should have to suppose that errors of record consist in systematic over-statements of deaths in small villages and equally systematic under-statements from large villages. Such an assumption would be quite

¹ See also Tables LXV and LXVI.

arbitrary without positive evidence and may be dismissed. I see no reason to attribute the result to errors of record.

We must therefore attempt to interpret these curious and interesting facts but, before doing so, it is well to ask whether the same or similar circumstances have been noticed before. Hankin¹, in 1905, pointed out a differential mortality rate in communities of different sizes; he contrasted village mortality with that of certain towns in Bombay. Hankin also called attention to the probable occurrence of a similar phenomenon in the Black Death. In Appendix I, I deal with such statistics as I could obtain bearing on the historic plagues; it will be found that these, so far as they go—unfortunately not very far—bear out Hankin's suggestion. They lend some support to a belief that we here have to deal with a general epidemiological phenomenon. It is to be noted that Hankin's work and my appendix are chiefly occupied with the contrast between urban and village mortality. In the case under discussion, the difference is found to be carried further and still to prevail amongst communities of the same general type.

This distinction is of epidemiological importance since the difference between a town and a village is not merely one of population, so that variation in rates of mortality might well depend upon factors which can hardly operate in village communities. In what follows I shall attend only to the latter class.

To throw light upon our problem we need first to consider how plague arises in a village and then how it spreads; let us examine these points in order.

I do not question that human epidemic plague (of bubonic type) in villages is derived from a rat epizootic by the mediation of rat fleas, particularly *X. cheopis*. Further, the evidence presented by Major Lamb and me (*op. cit.*), appeared to find its best interpretation on the hypothesis that village infections do not arise by recrudescence but from the introduction of infective material from outside. It must also be regarded as probable that neither transference by human contagion nor even by human parasites is *epidemiologically* important for the spread of village plague in the Punjab.

We shall, therefore, be justified in laying it down, as a fair working hypothesis supported by direct and indirect evidence, that in the great majority of the villages now being discussed epidemics have arisen in the following way:

¹ *Journ. Hyg.* v. (1905), p. 56.

At some point, or points, within the village an "effective" centre of rat plague has been established which then acts as a place of distribution for rat fleas some of which are infective. By "centre of effective occupation," I mean a place in which the rats *inhabiting* the house or hut have become infected and I distinguish an importation which has succeeded to this extent from the mere casual introduction of *materies morbi* not followed by an epizootic.

There are at least four ways in which an "effective" centre may arise:

- (1) By the introduction of an infected human being.
- (2) By the human importation, on the person or in the clothing, of infective fleas.
- (3) By the importation of infective fleas or infected rats in merchandise.
- (4) By the direct immigration of infected rats.

The last, having been specially considered by the Commission and found to have no apparent importance¹, need not detain us. With regard to the other methods², plainly, certain villages, owing to their geographical situation or some local peculiarity, might be specially prone to receive infection; but such factors have no necessary relation to size. The process initiated in the neighbourhood of an "effective" centre might be compared to a battery discharging shots—infective fleas—at random, the range being limited to a single house or group of dwellings and the rate of discharge increasing up to a certain limit. Given the establishment of one or more such primary "effective" centres, the question arises as to how the epidemic is spread through the community.

All the methods by which a centre is established *ab extra*, will still apply for the establishment of secondary centres within the community, and in addition we have to reckon with chances of spreading through the rat population by the transference of infective fleas from individual to individual. On the latter hypothesis, we should, perhaps, expect to be able to trace the lines of spread throughout the village starting from the initial centres and to find the infected areas, both epidemic and epizootic, steadily widening from week to week around these foci, until finally the distribution becomes approximately uniform.

¹ *Journ. Hyg. Plague No.*, vii. 1907, p. 907.

² I have purposely omitted certain entomological possibilities and considerations which are of moment but not, I think, in connection with the special topic discussed in this section.

An examination of the weekly spot-maps for Belgaum¹ does not entirely justify such anticipations; it is impossible to trace in them any regular process of extension from initial centres throughout the community although there is evidence of a restricted area of dissemination around each focus². The statistical evidence is equally inconclusive. Thus if plague tends to be uniformly spread through a village from the primary "effective" centres, we ought to find that the longer an outbreak endures the more nearly should the rates of mortality in different villages approximate, unless we assume that some factors correlated with absolute size arrest the extension. In other words, we ought to find that the difference between the rates of mortality in large and small villages should be greater in those only returning deaths in a single month than in villages affected in two or more months. I investigated this point, which was suggested by my colleague Dr Henderson Smith, in the way shown in Tables XXXV—XXXVIII. It cannot be said that these tables lend any real support to the idea that the difference in rates will diminish as we extend the duration of the epidemic. It cannot, of course, fairly be concluded from these results that the spread of an epidemic is not a consequence of a disseminated epizootic. The patchy character of the distribution may perhaps be accounted for in the following way. Let us suppose that at *a* and *b*, two widely separated spots, "effective" centres have been established. At and immediately around *a* and *b* there will be a considerable mortality among the rat population. This thinning of the ranks may induce an immigration of other rats so that the density of the rat population in any other places and therefore in the region between *a* and *b* will tend to be diminished. Some of the new-comers will be infected and, in course of time, the infected areas will extend, but the plague season may not last long enough for any distinct evidence of this to appear³.

In the meantime, new centres may be established at a distance from *a* and *b* by the casual emigrations of infected rats from the original points or owing to chance intercourse of the rats from *a* and *b* with individuals domiciled elsewhere.

Interesting as this problem may be, its solution is not germane to

¹ *Journ. Hyg.* x. (1910), pp. 446, etc.

² Cf. Brownlee, *Proc. Roy. Soc. Edinb.* xxxi. (1911), p. 262.

³ It is to be remarked that the daily movements of human beings about a village tends to vitiate any attempt to trace the course of dissemination from maps showing the dwelling houses of infected persons: see this *Journal*, Vol. vii. (1907), pp. 733, 860.

the present inquiry. What *is* material, however, seems to be the apparent fact that the extent of an epidemic must largely depend upon the number of "effective" centres established early in the epidemic. The diffusive power of plague is evidently not so great that one "effective" centre is sufficient to develop a wide-spread outbreak. A sun-dried expanse of brush-wood may be completely consumed if a fire is kindled at a single point within it, but, in moister circumstances, many independent conflagrations will be required. The latter half of the simile appears to correspond to the case of plague.

On the whole we may lay it down as a working hypothesis that the extent of an epidemic will be determined in the first place by the number of independent "effective" centres established, mainly by importation, at a relatively early stage in the plague season. The degree of effectiveness of these centres will further depend on the density of the surrounding population. Now, if we return to the enumeration of possible manners in which the primary centres may be established, it seems clear *a priori*, that, as a general rule, the number of centres will increase steadily with the population. Again, if the population density be greater in large than in small villages, the efficiency of the centres should increase with the population.

An associated factor would also seem to favour an increased rate of mortality in large villages. The nearer two "effective" centres are one to another the more favourable the conditions for producing plague, since the population of the area lying between the two centres will be, if the latter are not far apart, exposed to two fires. The average distance apart of a series of balls thrown at random upon a surface of known area will depend on the number of balls and the size of the area within which they must fall¹; consequently, if the area does not increase at the same rate as the number of centres, this circumstance would favour a relative increase of mortality in large villages. These *a priori* speculations lead to a result very different from the actual one. On the other hand, some considerations would point to a state of affairs qualitatively resembling what we do find.

Suppose the distribution of plague deaths *purely* random. Let the chance of a person dying from plague be p and of not dying q where

¹ I think, the average distance apart will vary as $\sqrt{\frac{A}{N}}$, where A is the number of units of area and N the number of imported foci of plague, but problems of this kind are intricate and the immediate practical importance of the matter hardly justifies a prolonged investigation.

$p + q = 1$. Then the proportional frequency of villages, each containing n inhabitants, which will report 0, 1, 2, etc., deaths is given by the terms of the expansion of $(p + q)^n$. Consequently the relative frequency of non-infected villages will be greater when n is small and the rate of mortality among the infected villages higher. The subjoined figures illustrate this

No. of deaths (to nearest unit)	Binomials	
	$1000(.01 + .99)^{100}$	$1000(.01 + .99)^{200}$
0	366	49
1	370	149
2	185	224
3	61	225
4	15	169
5	3	101
6	—	50
7	—	21
8	—	8
9	—	3
10	—	1
% non-infected villages ...	37.0	4.9
% deaths in infected villages	1.58	1.051

If, however, we consider any of the tables, *e.g.* Amritsar figures in Table XXXIV, it will be found that while p and q are of the order .03 and .97, n is of the order 460 and 1540 in the two groups. Thus we see that the proportion of non-infected villages in each group and the difference in rate of mortality between the groups transcend any possibility of simple explanation along these lines¹. We are accordingly forced to the conclusion that the observed results require the intervention of some other factor.

Let us return to modify our conception of the way in which the spread of plague through villages can be influenced. One fairly obvious modification is with respect to the density-factor. There must, one would suppose, be an upper limit to the degree of possible overcrowding and, although we might not expect it to have been reached in villages of the size contemplated here, we may suspect that such a circumstance has operated against the uniform increase of mortality rate with population. Perhaps the density of population increases with absolute size at a steadily diminishing rate, so that we might represent the change by some expression of the form $y = f(x)$, where y is the

¹ These remarks were suggested to me by Mr G. Udny Yule to whom I am further indebted for much valuable criticism of the first draft of this chapter.

density and x the gross population, the function being such that $y = k$ is an asymptote. But in order to account for the diminution in relative mortality as absolute size increases, it seems further necessary to suppose that (a) the number of initial centres does not increase at the same rate as the population, if we assume that the extent of an epidemic is determined by the number of such centres, or, (b), that, with an increase in population, factors limiting the epizootic extension gradually come into play. In order to decide between (a) and (b) we should have to ascertain from a large sample of villages (1) the density of population, and (2) the number of "effective" centres in the early weeks of the epizootic in each village. We have naturally no data on this scale of minuteness; some particulars can, however, be learned from the study of two Punjab villages, Dhand and Kasel.

The particulars which interest us are as follows:

Village	Population	Density per acre	Persons per house	Persons per room*	No. of houses plague-infected in 1st week of epizootic†
Dhand	1920	70	4.6	1.9	4
Kasel	3938	100	4.9	2.5	6†

* Calculated on the assumption that houses with more than two rooms have on the average 3.5 rooms each.

† Inclusive of a house with a human case but no plague-rats.

The information is of course defective from our point of view. It cannot, for instance, be said that the presence of human cases or even plague-rats in huts early in the epizootic, constitute the houses necessarily effective centres in my sense. Again, the measure of density employed is open to criticism. Still, *prima facie*, it would seem that the change both in density and number of initial foci does not keep pace with the absolute increase of the population. It would not, of course, be difficult to construct formulae which give changes in mortality rate with increase of population comparable to the observed results. We need merely suppose that the density increases at a diminishing rate and that the number of centres increases absolutely but not relatively to the growth of population. An illustration of the sort of graphical form that can be obtained in this way is given in an appendix (Appendix III), but practically such speculations have little value for the following reasons:

(1) There are no statistics by which to test any hypothetical formula.

(2) There is no proof that the extent of an epidemic depends upon the number of primary foci of imported plague.

It is indeed possible to seek an explanation upon different lines. For instance, it has been suggested that plague introduced among rats does not spread beyond the limits of the rat colony which received the infection from without. But, if village A with N inhabitants contains two distinct colonies of rats and village B with $\frac{N}{2}$ inhabitants only one colony, A is more likely to receive infection in one or other of its colonies than is B to suffer importation into its single colony. On the other hand, if B do get infected, the disease may be more widely disseminated through it than would be the case with a one-colony-infection in the larger village A. The principle here involved is precisely that which influenced relative mortality in favour of the small villages when we supposed the distribution of human plague to follow the simple binomial law (p. 79). I have given an arithmetical example above in dealing with that case; it will be interesting to consider the theory of the matter at a little more length in connection with the hypothesis just mentioned. It will, I believe, be found that any such interpretation is of doubtful validity.

Let p be the chance that plague will arise in a colony of rats and q the chance that it will not arise ($p + q = 1$). Let us suppose that in a village with N human inhabitants there will be a single colony of rats and in a village with mN inhabitants m such colonies. Let us further assume that infection cannot be conveyed from one rat colony to another and that the number of human plague-deaths varies directly with the number of plague-stricken rat colonies so that if k human deaths are associated with one infected colony, mk deaths occur when m colonies are infected. If T villages each containing mN inhabitants and m rat colonies be exposed to risk, the average rate of mortality in the infected villages will be $\frac{pk}{N(1-q^m)} = \frac{(1-q)k}{(1-q^m)N}$ ¹.

The average rate in villages of N inhabitants is, of course, $\frac{k}{N}$ and the ratio of the rates accordingly $\frac{1-q}{1-q^m}$ which diminishes as m increases and for a constant value of m diminishes as q increases².

¹ The total number of deaths

$$= Tk (mp^m + (m-1)mp^{m-1}q + \frac{(m-2)m(m-1)}{2!}p^{m-2}q^2 + \text{etc.}) = Tmpk(p+q)^{m-1} = Tmpk.$$

The population of the infected villages $= TmN(1-q^m)$.

$$\therefore \text{rate of mortality in infected villages} = \frac{pk}{N(1-q^m)}.$$

² Since it can be written $\frac{1}{1+q+q^2+q^3+\dots+q^{m-1}}$.

Now we have seen an illustration of this principle in the case of the ordinary binomial distribution obtained by assuming that human deaths from plague could be treated as simple independent occurrences. It was pointed out in this connection that the value of p deduced from the actual statistics was too large for the observed differences in mortality rates to be explicable along these lines. But the p of our present hypothesis will be larger than that of our former supposition since otherwise we should reach the absurd conclusion that the number of human plague deaths corresponding to an infected colony of rats is not less than the number of persons corresponding to a rat colony in general¹.

It is further to be remarked that the fall in rate of mortality as the populations of villages exposed to risk increase is exaggerated by the hypothesis that either plague in different colonies of rats or plague in different human beings are really independent. We have supposed that when a single colony is infected there will be k human deaths and when m colonies are infected mk deaths. But it is far more likely that of two villages each containing the same population and the same number of rat colonies, say, m rat colonies, and the former having m infected colonies and the latter only one, that the mortality in the first will be more than m times that of the second. Using the notation already employed we have for the total mortality on the hypothesis of independence :

$$Tk (mp^m + m(m-1)p^{m-1}q + \dots) \dots\dots\dots(1).$$

If we imagine some cumulative effect this will be changed into an expression of, *e.g.*, the form

$$Tk\alpha (\alpha^{-\frac{1}{m}} mp^m + \alpha^{-\frac{1}{m-1}} m(m-1)p^{m-1}q + \dots) \dots\dots\dots(2),$$

where α is greater than unity.

(2) will always be greater than (1) unless $m = 1$. Consequently the rate of mortality in infected villages of populations mN each will be greater than before.

I am accordingly unable to find a satisfactory explanation of the

¹ Let N = total population of a district, Nk_1 the number of human deaths from plague, Nk_2 the number of colonies of rats in the district, h the number of human deaths per infected colony of rats, p_1 the independent chance of a colony being infected and p_2 the chance of a human plague-death. $p_1 = \frac{Nk_1}{hNk_2} = \frac{k_1}{hk_2}$ and $p_2 = k_1$. If $\frac{k_1}{hk_2} < k_1$, $\frac{1}{k_2h_1} < 1$.

$\therefore k_2h > 1$. $\therefore h > \frac{1}{k_2}$. $\therefore$ no. of deaths per infected colony is greater than the average no. of persons per colony.

facts in this way. It has seemed worth discussing the more plausible hypotheses at some length because they afford an excellent illustration of the difficulty which has to be faced in attempting to give an account of a striking phenomenon¹. It would appear that we require the following additional statistics before an adequate theory can be propounded. We must know:

(1) Relation between the extent of an epidemic and the number of initial centres of plague.

(2) Relation between the human and rat population of villages of different sizes.

(3) Relation between the absolute population (human) and the density of population in villages.

We also require to know whether there is any relation between absolute size and prosperity, type of house, occupations of the villagers, etc. I am aware that such information is certainly difficult and perhaps impossible to obtain for an adequate number of villages. Without it, however, all I feel justified in concluding is that a difference can generally be observed between the rates of mortality in large and small villages from the region investigated and that this difference is no mere *simulacrum* or arithmetical will-o'-the-wisp, due to the employment of ratios. I conclude the section by expressing these results in summary form:

(1) *In nearly all the districts and epidemics studied the rate of mortality in small villages which became infected was higher than in large ones.*

(2) *This phenomenon cannot be regarded as a spurious effect due to the use of relative numbers or as an error of random sampling.*

(3) *If however the populations of villages not infected are included, there is no constant difference between the rates of mortality in large and small communities.*

V. *The influence of intra-seasonal variations in rainfall and temperature upon the rate of mortality in the villages under consideration.*

It is well known that the epidemic prevalence of plague is markedly seasonal, and the Commission have already studied the particular bearing of the general facts upon the epidemiology of plague in India². With the general conclusions arrived at in the report cited in the

¹ See further in Appendix IV.

² *Journ. Hygiene*, VIII. 1908, p. 288 *et seq.*; also *ibid.* x. 1910, p. 446, etc.; see also Sticker, *Abhandlungen aus der Seuchengeschichte*, etc., Bd. I. Part 2, pp. 238, etc.

footnote, the present data completely agree so that it is unnecessary to consider here that aspect of the subject. One topic, however, in connection with this matter has seemed worthy of notice on the present occasion. The constancy of the general type of seasonal prevalence in any one district shows that plague will only become epidemic when the meteorological conditions (or something highly correlated with these) fall within certain limits¹. It may be asked how far variations *within these limits* will alter the course and extent of the epidemic. Thus, we generally find that the mortality registered in any two epidemics is not precisely the same, often the difference is very considerable; can this be attributed to the differences in weather conditions which are found from year to year? Again, suppose that April is the month which contributes the greatest proportion of plague deaths, shall we find that the variations in the April quota from epidemic to epidemic can be brought into relation with changes in the average weather conditions of that month?

Complete data for answering these questions do not exist. In the first place we have the monthly plague figures separately recorded for only a few towns and villages; in the second, although we have numerous rainfall statistics and records of temperatures at a few places, no observations on percentage humidity are available for the districts under examination. Such data as are to be found have been summarised in Table XXXIX. Special details are given for Amritsar city in Table XL. Considering the relation of the course of several epidemics in Amritsar city and the temperature curve for Lahore, the conclusion to be drawn from a study of the facts is, I think, that both our questions must be answered in the negative. It does not seem that the relatively small changes from year to year in meteorological conditions within each part of the epidemic season can be regarded as of much importance as mortality factors. This general impression is confirmed by some applications of the method of correlation, all of which yielded negligible results. We must not hastily conclude from this that such variations are in fact without importance, but merely that their weight, in comparison with other factors, is not sufficient to reveal itself in the present kind of material. Study of the work published already by the Commission will convince the reader that the meteorological factor is of a complex nature. Its complete elucidation will require applications of the method of multiple correlation, for which the immediate statistics are not suitable.

¹ Some statistical points relative to the seasonal prevalence are examined in Appendix II *infra*.

VI. *On local differences in mortality.*

It will already have been noticed, in earlier tables, that the rate of plague mortality in villages infected in the same year, approximately equal in size and returning cases in the same cycle of months, exhibits not inconsiderable variations from district to district; within the same district, indeed, marked differences can be seen. This very important question will now be studied. Tables XLI-LVIII exhibit the rates of mortality in the different tehsils, or administrative subdivisions, of each district. Some notes on these tables are now given.

Hoshiapur. 1901-2. Una tehsil had a lower mortality than the others, but the whole outbreak was a small one.

1903-4. The same remarks apply to this year.

1904-5. Although a considerable number of villages in Una were at risk, the rate of mortality is still low.

1906-7. Una exhibits a lower rate of mortality than Garshankar or Dasuyah, but the same rate as Hoshiapur from which, paying attention to average size of village and length of exposure, it cannot be distinguished. The general experience of this district is that Una, which is separated from the rest of the district by a continuous range of hilly country, suffered less than the other tehsils. The difference is not however very striking.

Amritsar. 1902-3. Tarn-Tarn appears to have suffered more than either of the other tehsils, notably more than Ajnala notwithstanding the latter's lower average population.

1903-4. In this epidemic Ajnala is also relatively immune.

1904-5. Tarn-Tarn again takes the lead, the general picture being similar to that of 1902-3.

1905-6. Amritsar tehsil had many more infected villages than the other tehsils, also the heaviest rate of mortality.

1906-7. The features of 1903 and 1905 are reversed, the average population of Ajnala villages is however much less than that of the others. Evidently no general conclusion can be drawn as to the experience of this district which varied from year to year.

Rohtak. 1904-5. In view of its low average population, Jhajjar tehsil escaped lightly.

1906-7. The same remark applies again but very few Jhajjar villages were at risk.

Both the epidemics in Rohtak exhibit marked local divergences, a circumstance which must be taken into account when we remember

the apparent exception presented by this district to the general rule of higher mortality rates in smaller villages (*vide supra*). I do not however suggest that this is a complete explanation.

Mozaffarnagar. No appreciable local differences are to be found in this district.

Gujrat. 1903-4. Phalia tehsil exhibits an *enormously* greater mortality than the other tehsils, but also had a much larger number of villages at risk.

1904-5. No really significant differences in the local rates.

1906-7. This epidemic is the most interesting in the series because numerous villages were infected all over the district. The rate in Phalia is once again very much heavier than in Gujrat and, having regard to average population, appreciably heavier than in Kharian. Gujrat district suggested such marked local peculiarities in the incidence of plague that it was investigated in further detail. We notice in 1903-4, a decided maximum in the south-west part of the district. In 1904-5 the maximum has passed over in a north-east direction to the Gujrat tehsil. In 1905-6 less than 70 villages in all were infected, 32 in Gujrat, 12 in Kharian and 25 in Phalia, the respective rates being 1·3, 1·3 and 2·7%. In 1906-7, the wave sweeps round to the north-west, Kharian being deeply submerged. As I have said, this year is particularly important because the average populations and total numbers of villages infected are not very different in the three tehsils. To get more exact notions, a map was prepared. Circles were marked on a map of the district to show villages which had returned deaths corresponding to a rate of three times (or more) the average mortality for the epidemic. It will be noticed that a line roughly parallel to the main railway and seven or eight miles south-west of it would leave considerably more than half the "heavily infected" villages and rather less than half the total area below and to the south-west. The most striking concentration, however, relates to the big epidemic of 1906-7 alone. The large circle on the map has its centre at Dinga railway station and a radius of ten miles. Within its circumference were 122 infected villages the populations of which could be ascertained; in the rest of the district 729 villages were infected. The average mortality within this selected area reached the enormous figure of 17·3%, elsewhere it was less than half this. Further, only 6·6% of the villages reported deaths as early as February while 11·5% of the remainder were infected in this period. Some details appear in Tables LIX-LX. I think these differences are too considerable to be fairly attributed

either to errors of record or of random sampling. It would seem that some local influence was here at work which specially favoured plague ; of the nature of this factor, nothing can be gleaned from the statistics, so that it is not worth speculating here about the point, which, however, deserves attention. I think it must be admitted that some, unascertained, local peculiarities are of considerable significance, but that these factors are not necessarily constant from epidemic to epidemic. This will be seen from a general comparison of one district with another.

If we compare the districts for the three epidemics of a wide-spread character we have the following results :

District	Percentage mortalities in infected villages		
	1903-4	1904-5	1906-7
Gujrat	10.1	4.6	9.5
Rohtak	—	6.0	7.0
Amritsar	3.7	4.4	3.8
Mozaffarnagar	2.4	3.2	6.7
Hoshiapur	4.6	3.0	2.6

The differences as between the districts for the same epidemic are considerable. It will be noticed also that Gujrat and Rohtak, the districts within which we have seen the greatest local divergences in two out of the three epidemics were severely affected. Gujrat never takes a lower place than second in the order of severity. The general conclusions which seem to follow from the data here analysed can conveniently be grouped under six headings.

(1) The virulence of an epidemic in any one district varies greatly from year to year.

(2) This is partly due to some general phenomenon since the tendency is for the mortality to be high in all districts for a given epidemic or conversely.

(3) The influence is however partly local since the relative positions of the districts in respect of mortality rate are not quite the same from year to year.

(4) The importance of local factors is also demonstrated by the irregularity of incidence within any one district. An irregularity too great to be attributable to simple differences in average size, or length of exposure.

(5) The local factors, the sum of which makes in favour of a high rate of mortality, cannot be permanent in character since the subdivision

which has the heaviest mortality in one epidemic does not invariably occupy the same position in a subsequent outbreak.

(6) The rate of mortality is not necessarily heaviest in that subdivision which, judging from the number of infected villages, has plague most widely disseminated through it.

VII. *Summary of statistical conclusions.*

The detailed discussion of the conclusions here collected will be found in previous sections, together with the cautions necessarily to be borne in mind when weighing them (pp. 64, 65).

(1) Large villages tend to be infected earlier in the plague season than small ones.

(2) In villages of the same size total mortality is appreciably correlated with length of exposure to infection. The intensity of the relation, although quite appreciable, is not sufficient to account for the large differences in mortality rates noticed in villages of about the same size. There is some reason to think that villages infected before the months in which plague, owing to seasonal conditions, is most prevalent, do not suffer very appreciably more than villages not exposed until March or April.

(3) In nearly all cases, large *infected* villages suffer less than small ones.

(4) Plague does not occur at all in epidemic form during July, August and September in the districts analysed. The annual variations in temperature and rainfall observed during the epidemic season are not appreciably associated with variations in the rate of mortality.

(5) Differences in plague mortality in different districts, which are extremely marked, do not seem to be associated with climatic distinctions.

(6) Within any one district the variations in the rate of mortality observed in different subdivisions are considerable.

(7) A high rate of mortality in any subdivision is not necessarily accompanied by a wide dissemination of plague.

(8) There is no evidence that certain districts are *permanently* subject to a high rate of mortality. Local conditions making for an unusual degree of severity appear to vary from epidemic to epidemic.

(9) The rate of plague mortality in a village is seen to depend upon three factors, (a) length of exposure to infection, (b) number of inhabitants, (c) situation. Of these (c) is probably the most important; how it acts, however, cannot be determined from the present material.

VIII. *Some general remarks on the epidemiology of plague with special reference to village communities.*

In the following paragraphs, I shall epitomise some general considerations partly examined in the present and former reports. By adopting this course I hope to place the essential elements of the problem in a clearer light than was possible during the examination of a mass of statistical details.

The investigation of plague from the epidemiological side resolves itself into an attempt to answer three questions:

- (1) How does the disease enter a given country or district?
- (2) Having effected an entrance how does it maintain itself there?
- (3) What circumstances determine the transformation from endemic to epidemic prevalence and conversely?

(1) The majority of epidemiologists hold, I believe, that the origin of the present pandemic, the 1896 outbreak in Bombay, was probably plague ship-borne from Hong-Kong, but the point cannot be regarded as settled¹.

Any identification results from the weighing of probabilities somewhat delicately balanced and complete unanimity need not be expected. If we accept the hypothesis that plague always arises from pre-existent plague, a probable but not demonstrable supposition, it is to be remarked that its implantation evidently requires the co-operation of various factors the nature of which is still obscure. It is, perhaps, generally thought that the great pandemic revivals, *e.g.* that of the sixth century, the Black Death and the present outbreak depend in the last resort upon a special accession of virulence to the specific organism. This cannot, however, be a complete explanation since, for instance, the Provence plague of 1720-1, judging from its rate of mortality, was more virulent than the worst in our Indian experience; but it did not possess wide dispersive power. In other words, virulence and diffusibility are not interchangeable expressions. This part of the epidemiological subject does not, however, specially concern the phenomena of village plagues.

(2) That the intra-local spread of human plague is due to an epizootic cannot be seriously questioned so far as India is concerned.

Nevertheless we are not warranted in asserting that this method of

¹ See *Report of Indian Plague Commission*, 1901, v. p. 7; *The Present Pandemic of Plague*, by J. M. Eager, Washington, 1908; E. H. Hankin, *op. cit.*; G. Sticker, *op. cit.* Part 1, p. 354; W. J. Simpson, *Treatise on Plague*, 1905, pp. 66, etc.

spread has prevailed in all epidemics elsewhere, even when the type was predominatingly bubonic. For instance, in many parts of Europe, probably in English rural districts, the Black Death was chiefly bubonic plague, but I can find no trustworthy evidence that a rat epizootic was a factor in this particular pandemic.

In the Punjab, plague is maintained as a smouldering fire in some villages during the off-season. These villages generally exceed the average size, in some cases considerably. Apart from the fact that the likelihood of infection increases with size, other considerations tend to explain this, such for instance as the greater chance of persistent infection in an absolutely large than in a small population of rats.

It must of course be clearly understood that the statistical results obtained by Major Lamb and myself do not warrant us in asserting that *all* village plagues are started by importation from a large centre; but we are justified in affirming that this method of origin has been in all probability the most usual process. Hence, our answer to (2) is briefly—plague maintains itself in towns, not to any great extent in villages. Whether centres of occupation on an endemic level in the towns themselves require re-inforcement from a permanent focus of plague, is an epidemiological problem not needing consideration at this place.

(3) Our answer to (3) in broad outline rests upon remarkably full and concordant information. We see in the Punjab, in harmony with the experience of other countries and ages, that the first prerequisite of an epidemic is the realisation of certain conditions of temperature and humidity. In the case of villages having no plague in the off-season, the probability of an epidemic happening at all depends upon (α) size; *other things being equal*, the likelihood varies inversely as size. This inter-dependence is not, of course, direct, *i.e.* a village of population n is not just twice as likely to have an epidemic as one of population $\frac{1}{2}n$, but increase in the percentage of infected villages as the average population rises is substantial: (β) proximity to lines of transit; this being, in some but not all districts, evident when one studies the prevalence in villages near a railway. The *severity* of the epidemic in infected villages will be maximal when (*a*) importation occurs early in the plague season, (*b*) the village is a small one. I hope to have made it seem probable that this condition depends upon some material circumstance differentiating communities of different sizes. Since, so far as I am aware, no evidence of a comparable character has been collected with the object of determining whether the same rule

holds in the case of other epidemic diseases, such as cholera or enteric fever, it cannot be stated that (b) applies to plague alone. The historical evidence *suggests* that the rule may be of general application to epidemics of plague but cannot be regarded as of probative force. (c) We must also include, as a factor regulating severity, some local condition, constant for one epidemic but variable from epidemic to epidemic, which tends to raise or lower the mortality rate in contiguous hamlets irrespective of the numbers of their inhabitants.

It would naturally be expected that a severe epidemic would tend to exhaust the susceptible persons and rats so that severe and mild outbreaks would alternate. It might also be anticipated that dispersive and toxic powers would be, *to some extent*, associated so that epidemics affecting a large number of villages might have the highest rates of mortality. These surmises are only in part justified by the statistics collected in earlier chapters of the report (*e.g.* Table X). There is no marked regularity in the succession of severe and mild epidemics and the outbreak affecting the largest number of villages has not always—although usually—shown the highest mortality rate. It would not be safe to predict that a province seriously ravaged in one year will escape lightly in the following season.

With regard to the natures of factors comprised under (c), little can be said. It seems, however, improbable that climatic changes are responsible. I hope to have proved that variations both inter- and intra-local in the mortality rate are too large to be accounted for in this way. The same remark applies to changes from year to year; the exhaustion of susceptible persons and rats plays some, possibly a considerable part; climatic changes play a less, probably much less, important *rôle*; there remains a *tertium quid* which is not apt to be placed in evidence by statistical inquiries based on existing data.

I may be permitted to hope that the analyses communicated in this report have thrown light upon some points in the epidemiology of plague in a district containing scattered villages. I recognise that, in certain directions, it has been impossible to attain results of a character sufficiently definite to warrant one in attaching much importance to them. Even in these instances, however, it is possible that the labour has not been in vain because a first step towards the attainment of definite knowledge is a summary of the imperfections in data already collected.

In conclusion I would remark that the lamented death of Major George Lamb has deprived this report of much of the value it might

otherwise have possessed. The loss of his advice and criticism in the reduction of data for the most part prepared by him has been a serious blow, and I have particularly missed that wide knowledge of local circumstances which he had at his command.

APPENDIX I.

A note on town and village mortality rates from English plague records.

With the exception of an outbreak in "Cadwallader's Time" and a doubtful record for 1010-11, English plague experience is confined to a period of some 350 years, commencing with the importation of the Black Death, probably at Weymouth in 1348, and ending a few years after the Great Plague of London in 1665. I have brought together in this note such scanty evidence as I have found bearing upon the relative incidence of the disease upon different population groups.

(1) The Black Death. The rate of progress of the disease can be measured in London by a comparison of wills enrolled in the Hustings Court during 1348-9 with the average for other years. Such a comparison suggests that the mortality curve reached its maximum in April-May 1349. This does not correspond to the subsequent seasonal experience of London and may perhaps be explained by a tendency towards a pneumonic type. With regard to the actual figures, Robert of Avesbury asserts that upwards of 200 burials took place daily in Manny's cemetery before Easter, not to speak of interments elsewhere. Creighton infers that the maximum daily average may have reached 200, *i.e.* about that of the worst weeks in 1563. This would give a total mortality between 20,000 and 30,000, a rate of some fifty per cent. On the basis of Seebohm's data, it would appear that village mortality surpassed this figure (*vide infra*). Some indications point to the rate having been higher in Norwich than in London. Thus, before the Black Death, the proportion of men-at-arms levied on Norwich and London were in the ratio of 6 to 10 (writ of 1351 seemingly based on a pre-1349 standard). This is a rough measure of the ratio of the populations and confirmed by the numbers of ecclesiastical parishes in the two towns (120 in London, 60 in Norwich). Thirty years later, after the Black Death and several severe outbreaks subsequently, the populations were in the ratio of 17 to 100 (estimated from the Poll Tax returns of 1377). It may be that the relative decay of Norwich is

partly attributable to the ravages of plague which seems often to have been especially severe in the Eastern counties.

Turning now to the general rate of mortality all over England (see Table LXI), two methods of inquiry have been pursued, viz. an analysis of the number of institutions in different dioceses, and a study of Manor Court Rolls. Some of the figures relating to institutions of parochial clergy, due to the researches of Jessopp and Gasquet, appear in Table A. It would seem that in some districts the mortality was greater than sixty per cent. and there is no sign of a lower rate in the sparsely populated northern districts. With respect to village mortality, Jessopp's well-known results should be mentioned. In the manor of Cornard Parva, near the boundary of Essex and Suffolk, the tenants are estimated to have been less than fifty; in six months, 21 families were obliterated; sixty deaths are noticed in the Manor Rolls, this number not including all minors and dependents.

Similar evidence is available in the cases of Hunstanton and Croxford. Jessopp concludes that "during the year ending March 1350, more than half the population of East Anglia was swept away by the Black Death. If any one should suggest that *many more* than half died, I should not be disposed to quarrel with him" (Jessopp, p. 206). Seeböhm, who investigated the rolls of the manor of Winslow, states that 153 holdings changed hands during the plague; of 43 jurors who had served in 1346-8, 27 died in 1349, a mortality of over 60 per cent. (Seeböhm, p. 29). If these results are typical, it seems clear that the village rate was not less and may have been more than that of London¹.

I think the general impression to be obtained from a study of the fourteenth century pandemic is that, from the point of view of relative mortality, there is some similarity between its course and that of Indian experience; the similarity does not, however, extend to other epidemiological factors.

(2) When we come to the sixteenth and later centuries, at least one important epidemiological distinction is to be noticed. While some evidence, for example the Paston letter cited by Creighton (Creighton, p. 226), suggests that plague may have been endemic in country districts during the fifteenth century, there is no evidence of this being the

¹ The contemporary Simon de Corvino, in a poem on the Black Death, writes: "*Et nimis immensum sensere suburbia damnum*" (Haeser, III. p. 172). This may point to the mortality in small towns having been excessive, but the word *nimis* may have been introduced as a rhetorical expletive. I do not think we can, on the strength of this passage, cite de Corvino as an authority for the view that the Black Death was really more fatal in villages and small towns.

case in more recent times. The available facts suggest that village plagues in the sixteenth and seventeenth centuries arose by importation from towns. The question is whether the rates of mortality also contrasted. In Table LXII I have collected some of the less unreliable figures and the following records may be compared with them. In the years 1578-9, we have for London 4197 deaths (Jan. 1578-Dec. 1579), from April to December 1578, 3354 deaths. At Norwich from Aug. 20th 1578-Feb. 19th 1579, 4817 are recorded, 2335 English and 2482 aliens, giving a much heavier rate. Two thousand deaths are also reported in Yarmouth. In London during the plague year 1593, 17,844 was the total number of deaths, 10,662 from plague; in 1595 there were 3507 deaths from all causes so that the ratio of deaths in an ordinary year to deaths in a plague year would be 1:5.1. The burials in one parish of Shrewsbury during the plague year are in the ratio of 7.3:1, unity being the burials in an ordinary year. This suggests a rate certainly not below that of London. At the same period, more than 1100 deaths from plague are said to have occurred in the small city of Lichfield, but round figures of this sort are notoriously unreliable. In the town of Kirkoswald, plague appeared in 1598; the burials in that year were 583, in 1597 they numbered 42.

The famous epidemics of 1603, 1625, and 1665 tell the same story (see Table B). In the provinces, between the London plagues of 1625 and 1636, some towns experienced epidemics which would rank with the great London plagues in point of mortality rate, *e.g.* Newcastle (Creighton, p. 529).

(3) In Table LXIII I have collected data bearing upon the mortality rates of the epidemic in the South of France, 1720 (Sticker, 229). These results are indefinite but not irreconcilable with English experience.

Upon the whole, making due allowance for the fragmentary character of the material, it would appear probable that the rule deduced from the Punjab village statistics, *viz.* that the rate of plague mortality tends to increase as the absolute population of the infected community diminishes, may apply to European epidemics. If this be accepted, then, since it is doubtful whether the spread of the disease in the Black Death pandemic can be safely attributed to an epizootic, and it is probable that the material conditions of life in villages and small towns were inferior to those of the larger and more prosperous corporate cities, some support is afforded to the suggestion that material rather than numerical or purely epidemiological factors are responsible for the Punjab figures. It is unlikely that methods of sanitary administration, in the strict sense, can have had much to do with the results.

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APPENDIX II.

Note on the epidemic curves of Punjab districts.

While the general form of the epidemic curve is well known and has been frequently illustrated in the Advisory Committee's reports, it appeared worth while answering two questions.

- (1) Does the form vary from one district to another?
- (2) Is there any difference in the form of a severe as compared with that of a mild epidemic?

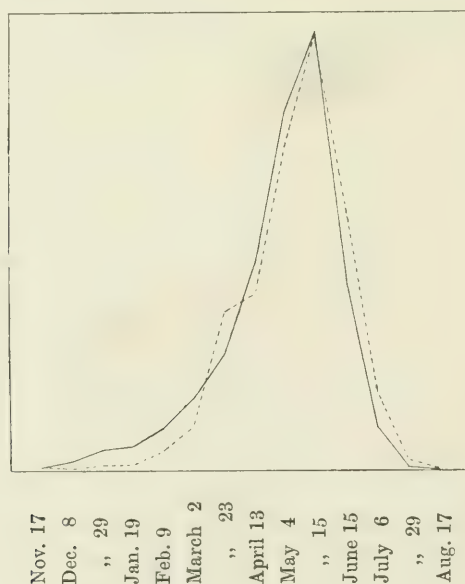


Fig. 3.

— Amritsar. - - - - - Gujrat.

I chose the 1906-7 epidemics for Amritsar and Gujrat districts as examples of severe outbreaks in a mainly agricultural (Gujrat) and partly urban (Amritsar) territory; 1905-6 Amritsar district illustrates the course of a relatively mild epidemic. The figures were obtained from the weekly official returns made to the Government of India. In

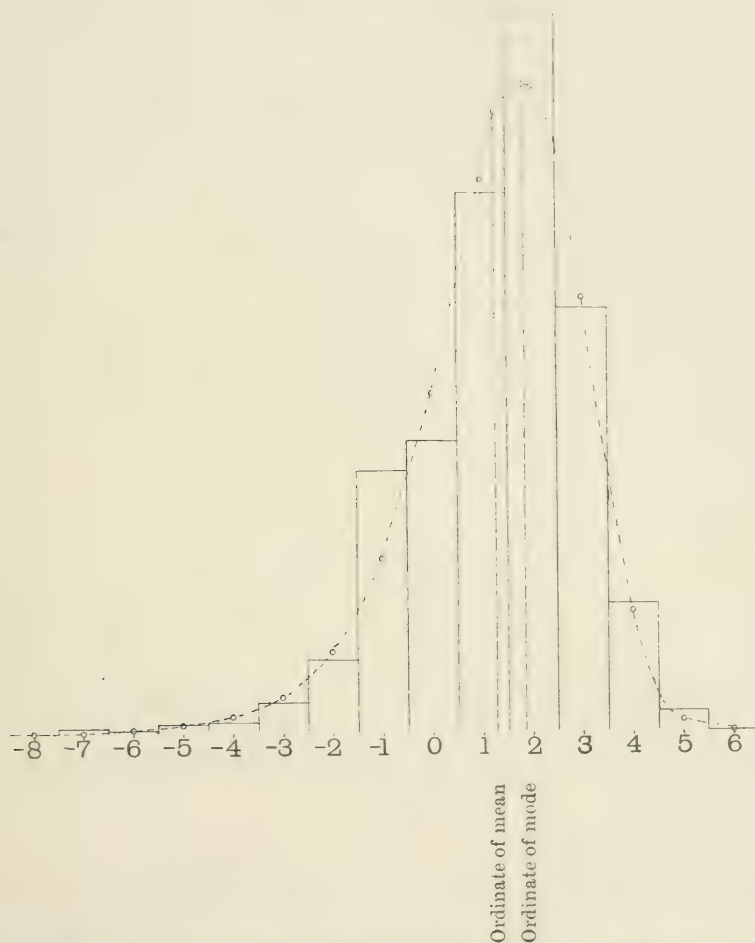


Fig. 4. Base unit, 3 weeks (the numerals refer to an arbitrary origin). Ordinate=no. of deaths from plague in each period of 3 weeks from Sept. 1906 to Sept. 1907, Gujrat. The rectangles represent the actual observations. The equation of the dotted curve is:

$$y = 1.1724 \left(1 + \frac{x^2}{(3.8854)^2} \right)^{-8.6661} \times e^{-19.5399 \tan^{-1} \frac{x}{3.8854}}$$

Origin at 6.2395.

the case of Amritsar, I have not entered the figures for Amritsar city when separately given.

As will be seen from the figures (Table LXIV) and diagram (Fig. 3) (in the diagram the two sets of figures are reduced to the same total) the course of the Gujrat epidemic almost exactly corresponds with that of Amritsar district. The asymmetry is marked, the fall being steeper than the rise. The minor epidemic of 1905-6 does not appear to differ in form from the wide-spread attacks.

The three sets of figures were analysed by Pearson's method. The Gujrat frequency proved to be of Type IV, Amritsar 1906-7 of Type I and Amritsar 1905-6 of Type VI. In each case the fit was poor although the Gujrat curve, which is reproduced (Fig. 4), is a tolerable representation of the course of events from the graphic standpoint. I think it is very doubtful whether the frequencies are homogeneous; there is certainly no ground for regarding them as even approximately symmetrical. In the result, so far as the data go, both questions proposed must be answered in the negative. The practical conclusion is that the factors which cut an epidemic short are more determinate than those responsible for its commencement—an inference warranted by all our other evidence.

APPENDIX III.

Note on possible interpretations of the curve of relative mortality.

It is not difficult to frame hypotheses which would account for changes in relative mortality with size similar to those discussed in the text. The following is given as an elementary illustration; it is to be clearly understood that the assumptions made do not rest upon any reliable basis.

If the two factors which influence the rate of mortality be (1) number of initial plague centres, (2) density of population.

If, further, (1) increases more slowly than the absolute population, and if (2) increases with the population to a constant value, then

$$y = A\alpha^{\frac{k}{n+1}}\beta^{-\frac{1}{n}} \dots\dots\dots(1)$$

is a possible form, where y = the rate of mortality, n the number of inhabitants per village and α , β , k and A are constants.

If, for simplicity, we put $\alpha = \beta$ we have

$$y = A\alpha^{\frac{n(k-1)-1}{(n+1)n}} \dots\dots\dots(2).$$

Differentiating (2) we find that y has a maximum when $n = \frac{-1 \pm \sqrt{k}}{1-k}$.

The diagram (Fig. 5) which gives part of the curve $y = 10^{\frac{3n-1}{(n+1)n}}$ indicates a state of affairs suggestive of the actual facts. The nature of the assumptions does not warrant us in attaching much significance to the results. The subject is, however, of interest and might be worth further investigation along these lines.

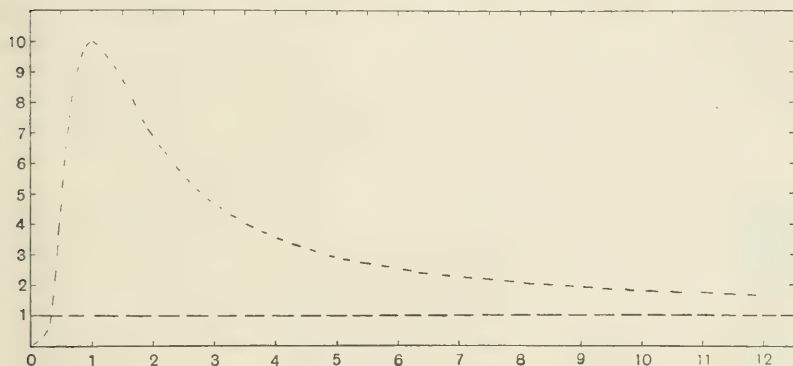


Fig. 5.

TABLE I.

РОМТАК 1905.			РОМТАК 1905.		
<i>Populations 200—250.</i>			<i>Populations 650—700.</i>		
Population	Actual number of deaths	Calculated number	Population	Actual number of deaths	Calculated number
200	20	20·29	656	1	59·79
209	75	21·21	664	130	60·52
211	5	21·41	663	60	60·43
216	6	21·92	669	9	60·98
224	21	22·73	675	67	61·52
231	4	23·44	677	173	61·71
1291	131	131·00	680	11	61·98
<i>Populations 600—650.</i>			681	38	62·07
603	10	22·77	5365	489	489·00
606	23	22·89	<i>Populations 700—750.</i>		
609	38	23·00	729	33	45·31
612	41	23·12	725	20	45·06
622	6	23·49	723	49	44·94
634	39	23·95	721	52	44·81
637	20	24·06	710	61	44·13
645	31	24·36	706	29	43·88
645	4	24·36	705	70	43·82
5613	212	212·00	741	44	46·05
<i>Populations 950—1000.</i>			5760	358	358·00
951	27	77·71	<i>Populations 750—800.</i>		
954	105	77·96	751	103	39·73
956	36	78·12	759	18	40·15
957	98	78·21	766	31	40·52
964	72	78·78	782	48	41·37
974	72	79·59	796	29	42·11
981	73	80·16	796	17	42·11
997	149	81·47	4650	246	245·99
7734	632	632·00			

TABLE II.

РОНТАК 1905.

<i>Populations 1400—1500.</i>			<i>Populations 1650—1700.</i>		
Population	Actual number of deaths	Calculated number	Population	Actual number of deaths	Calculated number
1409	92	73·05	1658	186	115·33
1415	161	73·36	1660	99	115·47
1431	176	74·19	1661	60	115·54
1432	90	74·25	1671	178	116·23
1445	12	74·92	1681	143	116·93
1467	28	76·06	1686	16	117·28
1468	85	76·11	1698	195	118·11
1474	21	76·43	1698	56	118·11
1478	10	76·63			
13,019	675	675·00	13,413	933	933·00

TABLE III.

РОНТАК 1905.

Populations 350—400.

Population	Actual number of deaths	Calculated number
354	4	22·32
356	33	22·45
357	18	22·51
359	97	22·64
362	7	22·83
363	3	22·89
364	21	22·95
369	7	23·27
372	37	23·46
380	5	23·96
383	19	24·15
391	31	24·66
393	6	24·78
396	30	24·97
399	35	25·16
5598	353	353·00

Populations 250—300.

266	5	12·92
267	2	12·97
267	11	12·97
269	16	13·07
273	1	13·26
275	8	13·36
277	13	13·46
286	25	13·89
292	26	14·19
296	18	14·38
299	24	14·53
3067	149	149·00

РОНТАК 1905.

Populations 400—500.

Population	Actual number of deaths	Calculated number
400	207	39·34
410	33	40·32
413	15	40·61
418	38	41·11
419	3	41·20
423	33	41·60
435	55	42·78
437	30	42·97
443	23	43·57
450	6	44·25
453	21	44·55
457	91	44·94
485	31	47·70
488	40	47·99
499	26	49·07
6630	652	652·00

Populations 500—550.

511	50	18·52
513	20	18·59
517	13	18·74
525	8	19·03
526	4	19·06
526	18	19·06
3118	113	113·00

TABLE IV.

RoHTAK 1905.			RoHTAK 1905.		
<i>Populations 1150—1200.</i>			<i>Populations 1000—1100.</i>		
Population	Actual number of deaths	Calculated number	Population	Actual number of deaths	Calculated number
1155	103	77·76	1004	93	52·80
1160	45	78·09	1014	96	53·32
1171	19	78·83	1031	43	54·22
1173	87	78·97	1035	38	54·43
1177	98	79·24	1042	11	54·79
1178	165	79·30	1059	6	55·69
1181	61	79·51	1059	79	55·69
1188	57	79·98	1060	3	55·74
1191	78	80·18	1064	128	55·95
1192	63	80·25	1077	115	56·63
1193	54	80·31	1084	96	57·00
1197	123	80·58	1086	14	57·11
14,156	953	953·00	1092	10	57·42
			1095	8	57·58
			1096	96	57·63
			15,898	836	836·00
<i>Populations 1200—1300.</i>			<i>Populations 1100—1150.</i>		
1298	1	77·45	1102	65	66·62
1296	76	77·33	1104	59	66·74
1297	6	77·39	1112	102	67·23
1285	39	76·68	1113	26	67·29
1285	7	76·68	1141	49	68·98
1236	275	73·75	1141	125	68·98
1228	119	73·28	1144	49	69·16
1214	82	72·44	7857	475	475·00
10,139	605	605·00			

TABLE V.

AMRITSAR 1904.			AMRITSAR 1903.		
<i>Populations 700—750.</i>			<i>Populations 700—750.</i>		
Population	Actual number of deaths	Calculated number	Population	Actual number of deaths	Calculated number
750	6	15.49	750	33	33.11
750	16	15.49	750	6	33.11
715	21	14.77	719	10	31.75
719	9	14.85	740	15	32.67
714	22	14.75	711	14	31.39
715	1	14.77	727	30	32.10
730	34	15.08	727	55	32.10
711	8	14.69	746	27	32.94
734	1	15.16	703	58	31.04
746	56	15.41	721	50	31.83
749	18	15.47	716	49	31.61
721	11	14.89	727	18	32.10
716	19	14.79	707	55	31.22
727	2	15.02	715	60	31.57
707	17	14.60	721	40	31.83
715	4	14.77	749	11	33.07
707	29	14.60	713	29	31.48
743	8	15.35	707	10	31.22
729	3	15.05	726	41	32.05
13,798	285	285.00	743	30	32.81
			14,518	641	641.00

TABLE VI.

GUJRAT 1907.			GUJRAT 1907 (<i>cont.</i>)		
<i>Populations 450—500.</i>					
Population	Actual number of deaths	Calculated number	Population	Actual number of deaths	Calculated number
467	1	46.79	475	2	47.59
496	73	49.70	499	21	50.00
479	37	47.99	493	70	49.40
467	34	46.79	499	19	50.00
476	27	47.69	456	20	45.69
474	4	47.49	457	188	45.79
460	28	46.09	490	23	49.10
469	2	46.99	453	18	45.39
491	29	49.20	458	54	45.89
459	2	45.99	497	170	49.80
459	2	45.99	461	68	46.19
498	6	49.90	475	41	47.59
467	17	46.79	466	60	46.69
454	35	45.49	466	75	46.69
463	32	46.39	473	33	47.39
472	42	47.29	478	114	47.89
496	134	49.70	490	22	49.10
468	100	46.89	500	21	50.10
458	18	45.89	498	45	49.90
467	174	46.79	462	64	46.29
			456	23	45.69
			19,442	1948	1948.01

TABLE VII.

GUJRAT 1907.			GUJRAT 1907 (cont.)		
<i>Populations 750—800.</i>			Population	Actual number of deaths	Calculated number
Population	Actual number of deaths	Calculated number			
794	11	90.81	769	77	87.95
771	7	88.18	767	270	87.73
767	98	87.73	760	70	86.93
768	103	87.84	753	66	86.12
758	5	86.70	770	43	88.07
775	145	88.64	797	31	91.16
787	62	90.01	778	67	88.98
785	135	89.79	786	89	89.90
779	220	89.10	794	16	90.81
755	295	86.35	752	60	86.01
781	62	89.33	796	66	91.04
764	27	87.38	775	94	88.64
			785	96	89.79
			19,366	2215	2214.99

TABLE VIII.

HOSHIARPUR 1902.			HOSHIARPUR 1907.		
<i>Populations 700—750.</i>			<i>Populations 700—750.</i>		
Population	Actual number of deaths	Calculated number	Population	Actual number of deaths	Calculated number
722	21	20.46	739	2	21.97
707	27	20.03	712	15	21.17
745	42	21.11	748	1	22.24
717	31	20.32	729	14	21.68
742	4	21.02	746	19	22.18
729	4	20.65	709	20	21.08
748	36	21.19	747	9	22.21
703	7	19.92	739	13	21.97
746	30	21.14	742	62	22.06
739	21	20.94	711	14	21.14
749	5	21.22	725	116	21.56
8047	228	228.00	711	1	21.14
			722	2	21.47
			707	6	21.02
			711	14	21.14
			706	34	20.99
			745	36	22.15
			734	11	21.83
			13,083	359	389.00

TABLE IX.

General Statistical Account of the five Districts.

RELIGIONS.

District	Total population		Hindu	Percentage of total	Sikh	Percentage of total	Mohammedan	Percentage of total	Others	Percentage of total
Amritsar	1,023,828	100·0	280,985	27·4	264,329	25·8	474,976	46·4	3538	·4
Rohtak	630,672	100·0	533,723	84·6	94	—	91,687	14·6	5168	·8
Gujrat	750,548	100·0	69,346	9·2	24,893	3·3	655,838	87·4	471	·1
Hoshiarpur	989,782	100·0	603,710	61·0	71,126	7·2	312,958	31·6	1988	·2
Mozaffarnagar	877,188	100·0	606,833	69·2	280	—	255,292	29·1	14,783	1·7

OCCUPATIONS.

District	Pasture and Agricultural	Percentage of total	Unskilled Labour (not Agricultural)	Percentage of total	Provision of Food, Drink, &c.	Percentage of total	Provision of Textile Fabrics, Furs, &c.	Percentage of total	Personal and Household Services, &c.	Percentage of total	Others	Percentage of total
Amritsar	409,690	40·0	19,005	1·9	62,242	6·1	101,467	9·9	165,611	16·2	265,813	25·9
Rohtak	385,194	61·1	7626	1·2	20,567	3·3	35,242	5·6	40,127	6·3	141,916	22·5
Gujrat	476,177	63·4	14,913	2·0	27,351	3·6	47,614	6·3	45,130	6·1	139,363	18·6
Hoshiarpur	594,180	60·0	20,898	2·1	29,296	3·0	84,354	8·5	60,974	6·2	200,080	20·2
Mozaffarnagar	449,260	51·2	98,804	11·3	50,143	5·7	53,109	6·1	86,200	9·8	139,672	15·9

TRADE.

There are no District Statistics of imports and exports and the general information as to the trade of separate districts is meagre.

Amritsar	exports wheat, shawls, carpets, cotton goods, jewellery and brass vessels, and imports (partly for re-export) grain, pulses, sugar, oil, salt, tobacco, raw cotton, cotton goods and yarn, wool, silk, shawls, blankets, metals, hardware, glass and dyes.
Rohtak	exports cereals.
Gujrat	exports wheat, millet, oil-seeds, oil, <i>ghi</i> , wool, cotton, cotton goods and hides, and imports piece goods, iron, sugar, salt, rice, wool, brass vessels, spices and dyes.
Hoshiarpur	exports rice, grain, wheat, barley, sugar, hemp and other fibres, tobacco, indigo, cotton and lac, and imports cotton and cotton yarn, piece goods, millet, cattle, dyes, salt, sugar, tobacco, kerosine, and oil-seeds.
Mozaffarnagar	exports wheat, raw sugar, barley, millet, rice and oil-seeds.

TABLE X.

Particulars of all Epidemics in each District.

District	Year	Total population	Number of villages infected	Total plague mortality	Death rate per cent.	Average population of infected villages
Hoshiarpur	1898	17,438	16	439	2.52	1089.9
„	1899	3701	4	9	.24	925.3
„	1900	9636	11	50	.52	876.0
„	1901	42,790	45	444	1.04	950.9
„	1902	404,430	476	11,688	2.89	849.6
„	1903	No data.				
„	1904	572,120	856	26,067	4.56	668.4
„	1905	619,241	952	19,131	3.09	650.5
„	1906	228,728	293	4125	1.80	780.6
„	1907	598,384	892	15,707	2.63	670.8
Gujrat	1902	72,200	38	551	.76	1900.0
„	1903	87,724	58	1794	2.05	1512.5
„	1904	277,151	305	23,785	8.58	908.7
„	1905	231,535	262	9782	4.22	883.7
„	1906	120,219	60	1207	1.00	2000.7
„	1907	560,579	853	51,000	9.10	657.2
Amritsar	1902	259,583	59	2039	.79	4399.7
„	1903	700,040	480	27,285	3.90	1458.4
„	1904	671,548	430	19,475	2.90	1561.7
„	1905	846,148	644	30,719	3.63	1313.9
„	1906	502,199	273	7911	1.58	1839.6
„	1907	796,099	591	24,743	3.11	1347.0
Mozaffarnagar	1902	25,581	2	4	.02	12,790.5
„	1903	73,944	23	782	1.06	3215.0
„	1904	257,455	128	5486	2.13	2011.4
„	1905	545,689	316	16,246	2.98	1726.9
„	1906	174,409	68	3077	1.76	2564.8
„	1907	753,403	590	49,909	6.62	1277.0
Rohtak	1904	147,918	56	2990	2.02	2641.4
„	1905	491,746	284	28,802	5.86	1731.5
„	1906	121,872	31	1948	1.60	3931.4
„	1907	470,442	249	31,476	6.69	1889.3

TABLE XI.

AMRITSAR.

Epidemic of 1903.

Month of 1st infection	Total population	Number of deaths	Number of villages	Death rate per 1000	Mortality ratio March=1·00	Mean population of villages
Dec. '02	69,362	4324	52	62·34	1·09	1333·88
Jan.	71,890	3782	71	52·61	·92	1012·54
Feb.	84,558	5071	72	59·97	1·05	1174·42
March	104,639	5969	95	57·94	1·00	1101·46
April	89,723	4657	86	51·90	·91	1043·29
May	89,208	2533	86	28·39	·50	1037·30
June	9436	194	14	20·56	·36	674·00
	518,816	26,530	476	51·14	·883	1089·95

Epidemic of 1904.

Dec. '03	3879	56	2	14·44	·26	1939·5
Jan.	45,000	1715	20	38·11	·70	2250·0
Feb.	34,207	2103	23	61·48	1·12	1487·26
March	89,296	4886	70	54·72	1·00	1275·66
April	223,926	7980	207	35·64	·65	1081·77
May	92,137	1217	100	13·21	·24	921·37
June	1879	10	4	5·32	·01	469·75
	490,324	17,967	426	36·64	·670	1151·00

Epidemic of 1905.

Dec. '04	110,364	5826	75	52·79	1·13	1471·5
Jan.	120,386	5671	118	47·11	1·01	1020·2
Feb.	102,158	5153	95	50·44	1·08	1075·3
March	139,296	6510	128	46·74	1·00	1088·3
April	112,273	4551	125	40·54	·87	898·2
May	77,592	1729	93	22·28	·48	834·3
June	2855	38	6	13·31	·28	475·8
	664,924	29,478	640	44·33	·948	1038·94

TABLE XII.

AMRITSAR.

Epidemic of 1906.

Month of 1st infection	Population	No. of deaths	No. of villages	Death rate per 1000	Mortality ratio March=1·00	Mean population of villages
Dec. '05	2463	68	2	27·61	1·07	1231·5
Jan.	18,697	541	12	28·94	1·12	1558·1
Feb.	15,962	415	10	26·00	1·01	1596·2
March	64,953	1676	38	25·80	1·00	1709·3
April	97,009	2333	97	24·05	·93	1000·1
May	96,163	693	92	7·21	·28	1045·3
June	21,967	78	16	3·55	·14	1372·9
	317,214	5804	267	18·30	·709	1188·07

Epidemic of 1907.

Dec. '06	53,045	2701	42	50·92	1·20	1263·0
Jan.	35,877	1203	30	33·60	·79	1193·6
Feb.	60,723	2553	50	42·04	·99	1214·5
March	158,190	6732	128	42·56	1·00	1235·9
April	213,221	7528	197	35·31	·83	1082·3
May	103,421	2851	129	27·57	·65	801·7
June	17,965	260	27	14·47	·34	665·4
	642,442	23,828	603	37·09	·871	1065·4

TABLE XIII.

GUJRAT.

Epidemic of 1904.

Month of 1st infection	Total population	No. of deaths	No. of villages	Death rate per 1000	Mortality ratio March=1·00	Mean population of villages
Dec. '03	13,653	912	10	66·79	·42	1365·3
Jan.	3808	1051	4	276·00	1·75	952·0
Feb.	16,529	1790	14	108·29	·69	1180·6
March	58,132	9185	68	158·00	1·00	854·9
April	98,336	9719	143	98·83	·63	687·7
May	30,568	842	46	27·55	·17	664·5
June	12,327	144	16	11·68	·07	770·4
July	620	1	1	1·61	·01	620·0
	233,973	23,644	302	101·05	·64	774·8

Epidemic of 1905.

Dec. '04	11,359	821	14	72·28	1·31	811·4
Jan.	8998	594	16	66·01	1·20	562·4
Feb.	8685	629	13	72·42	1·31	668·1
March	48,480	2675	52	55·18	1·00	932·3
April	70,686	3697	93	52·30	·95	760·1
May	46,857	833	61	17·78	·32	768·2
June	5027	28	11	5·57	·10	457·0
July	—	—	—	—	—	—
	200,092	9277	260	46·36	·84	769·6

Epidemic of 1907.

Dec. '06	28,196	1741	16	61·75	·49	1762·3
Jan.	15,824	1909	20	120·63	·95	791·2
Feb.	40,836	5956	56	145·88	1·15	729·2
March	120,790	15,299	165	126·66	1·00	732·1
April	190,674	19,744	294	103·55	·82	648·6
May	102,997	4984	224	48·39	·38	459·8
June	26,014	563	67	21·64	·17	388·3
July	3805	39	9	10·25	·08	422·8
	529,136	50,235	851	94·94	·75	621·8

Village Plague

TABLE XIV.

HOSHIARPUR.

Epidemic of 1902.

Month of 1st infection	Total population	Number of deaths	Number of villages	Death rate per 1000	Mortality ratio (March=1·00)	Mean population of villages
Dec. '01	104,392	4543	116	43·52	3·07	899·9
Jan.	84,300	2720	89	32·27	2·28	947·2
Feb.	71,816	2342	98	32·61	2·30	732·8
March	67,473	957	79	14·18	1·00	854·1
April	47,918	896	52	18·70	1·32	921·5
May	22,864	176	34	7·70	·54	672·5
June	905	3	2	3·31	·23	452·5
July	4762	51	6	10·71	·76	793·7
	404,430	11,688	476	28·90	2·038	849·64

Epidemic of 1904.

Dec. '03	—	—	—	—	—	—
Jan.	62,900	2506	61	39·84	·81	1031·15
Feb.	96,191	5805	92	60·35	1·22	1045·55
March	110,282	5445	136	49·37	1·00	810·9
April	136,753	7047	249	51·53	1·04	549·1
May	127,600	4595	243	36·01	·73	525·1
June	30,176	446	62	14·78	·30	486·7
July	8218	223	13	27·14	·55	632·15
	572,120	26,067	856	45·56	·923	668·36

TABLE XV.

HOSHIARPUR.

Epidemic of 1905.

Month of 1st infection	Total population	Number of deaths	Number of villages	Death rate per 1000	Mortality ratio (March=1·00)	Mean population of villages
Dec. '04	71,162	3534	98	49·66	1·59	726·14
Jan.	194,770	6495	230	33·35	1·07	846·83
Feb.	87,999	2391	119	27·17	·87	739·49
March	82,962	2585	144	31·16	1·00	576·13
April	96,801	2640	186	27·27	·88	520·44
May	49,109	977	96	19·89	·64	511·55
June	29,774	423	65	14·21	·46	458·06
July	6664	86	14	12·91	·41	476·00
	619,241	19,131	952	30·89	·991	650·46

Epidemic of 1907.

Dec. '06	73,603	2475	99	33·63	1·14	743·46
Jan.	119,808	3346	123	27·93	·95	974·05
Feb.	96,984	3069	139	31·64	1·06	697·73
March	133,924	3937	224	29·40	1·00	597·88
April	113,387	2248	192	19·83	·67	590·56
May	39,691	569	90	14·34	·49	441·01
June	18,463	108	20	5·85	·20	923·15
July	2748	15	5	5·46	·19	549·60
	598,608	15,767	892	26·35	·893	671·10

TABLE XVI.
MOZAFFARNAGAR.

Epidemic of 1904.

Month of 1st infection	Total population	Number of deaths	Number of villages	Death rate per 1000	Mortality ratio March=1·00	Mean population of villages
Dec. '03	19,419	651	6	33·52	1·25	3236·5
Jan.	18,315	616	7	33·63	1·25	2616·4
Feb.	38,581	1331	20	34·50	1·28	1929·1
March	58,233	1567	30	26·91	1·00	1949·1
April	57,720	822	46	14·24	·53	1254·8
May	10,954	138	9	12·60	·47	1217·1
June	7663	24	7	3·13	·12	1094·7
July	—	—	—	—	—	—
	210,885	5149	125	24·42	·908	1687·08

Epidemic of 1905.

Dec. '04	137,988	4975	62	36·05	1·17	2225·6
Jan.	82,538	3012	48	36·49	1·18	1719·5
Feb.	39,781	1402	29	35·24	1·14	1371·8
March	126,554	3908	82	30·88	1·00	1543·34
April	67,114	2029	54	30·23	·98	1242·85
May	34,343	546	34	15·90	·51	1010·1
June	3069	7	4	2·28	·07	767·3
July	—	—	—	—	—	—
	491,387	15,879	313	32·31	1·046	1569·93

TABLE XVII.

MOZAFFARNAGAR.

Epidemic of 1906.

Month of 1st infection	Total population	Number of deaths	Number of villages	Death rate per 1000	Mortality ratio (March=1·00)	Mean population per village
Dec. '05	24,829	473	9	19·05	·66	2758·8
Jan.	3428	214	2	62·43	2·15	1714·0
Feb.	17,309	1014	8	58·58	2·02	2163·6
March	28,423	825	18	29·03	1·00	1579·1
April	27,346	298	19	10·90	·38	1439·3
May	15,895	85	8	5·35	·18	1986·9
June	2699	1	1	·37	·013	2699·0
July	—	—	—	—	—	—
	119,929	2910	65	24·26	·836	1845·06

Epidemic of 1907.

Dec. '06	83,580	3746	41	44·82	·503	2038·5
Jan.	87,134	7504	50	86·12	·95	1742·7
Feb.	133,234	10,522	81	78·97	·88	1644·9
March	145,205	13,126	133	90·40	1·00	1091·8
April	160,761	9665	174	60·12	·67	923·9
May	80,158	1952	99	24·35	·27	809·7
June	9022	122	10	13·52	·15	902·2
July	—	—	—	—	—	—
	699,094	46,637	588	66·77	·739	1188·94

Village Plague

TABLE XVIII.

ROHTAK.

Epidemic of 1905.

Month of 1st infection	Total population	Number of deaths	Number of villages	Death rate per 1000	Mortality ratio (March=1·00)	Mean population of villages
Dec. '04	63,620	3500	25	55·01	·64	2544·8
Jan.	97,979	7387	41	75·39	·88	2389·7
Feb.	67,554	4588	43	67·92	·79	1571·0
March	82,796	7080	57	85·51	1·00	1452·6
April	114,258	4687	95	41·02	·48	1202·7
May	30,624	401	19	13·09	·15	1611·8
June	1095	8	1	7·31	·09	1095·0
Total	457,926	27,651	281	60·38	·71	1629·6

Epidemic of 1907.

Dec. '06	38,129	3603	11	94·50	1·16	3466·3
Jan.	29,508	3141	11	106·40	1·58	2228·0
Feb.	57,078	4400	26	77·09	·96	2195·3
March	169,885	13,799	85	81·23	1·00	1998·7
April	96,351	5220	73	54·18	·67	1319·9
May	42,900	643	35	14·99	·18	1225·7
June	4290	43	6	10·02	·12	715·0
Total	438,141	30,849	247	70·40	·88	1753·6

TABLE XVIII A.

Particulars of villages infected in December or earlier and grouped together in Tables XI—XVIII.

HOSHIARPUR 1907.

Month of 1st infection	Population	Deaths	No. of villages	Mortality %	Average population of villages
July	7010	283	7	4·04	905·8
August	434	20	3	4·61	144·7
September	764	3	2	·39	382·0
October	4803	142	8	2·96	600·4
November	28,136	832	35	2·96	803·9
December	32,456	1195	44	3·68	737·6
Total	73,603	2475	99	3·36	743·5

AMRITSAR 1907.

September	1409	72	2	5·11	704·5
October	3469	121	3	3·49	1156·3
November	17,175	1039	14	6·05	1226·8
December	30,992	1469	23	4·74	1347·5
Total	53,045	2701	42	5·09	1263·0

MOZAFFARNAGAR 1907.

October	12,266	515	7	4·20	1752·3
November	21,436	799	8	3·73	2679·5
December	49,878	2432	26	4·88	1918·4
Total	83,580	3746	41	4·48	2036·6

TABLE XIX.

Relation between length of exposure and number of deaths in the whole epidemic.

Population group	Number of villages	Number of deaths	Mean deaths per village	Population	Mean population of villages	Standard deviation of population	Coefficient of variation	r
Hoshiarpur 1902 :								
10—200	46	380	8.3	5416	117.7	56.5	47.98	+ .44 ± .08
130—270	57	576	10.1	11,763	206.4	40.2	19.47	+ .41 ± .07
250—350	57	565	9.9	17,067	299.4	28.6	9.56	+ .37 ± .08
350—450	56	683	12.2	22,099	394.6	29.0	7.36	+ .40 ± .08
450—550	41	797	19.4	20,365	496.7	28.5	5.73	+ .29 ± .10
525—675	58	987	17.0	34,641	597.3	42.4	7.10	+ .31 ± .08
625—775	41	905	22.1	28,626	698.2	50.2	7.19	+ .47 ± .08
700—900	51	1318	25.8	41,009	804.1	55.7	6.92	+ .46 ± .07
800—1000	48	1544	32.2	43,073	897.4	61.3	6.83	+ .29 ± .09
875—1125	50	1676	33.5	49,710	994.2	70.5	7.09	+ .38 ± .08
Hoshiarpur 1905 :								
75—125	48	357	7.4	4765	99.3	16.0	16.07	- .06 ± .10
125—175	62	481	7.8	9228	148.8	15.0	10.09	+ .41 ± .07
175—225	73	671	9.2	14,818	203.0	14.5	7.14	+ .36 ± .07
225—275	75	776	10.3	18,642	248.6	15.7	6.32	+ .21 ± .07
275—325	65	494	7.6	19,438	299.0	14.7	4.91	+ .14 ± .08
325—375	76	1135	14.9	26,799	352.6	15.0	4.24	+ .34 ± .07
375—425	50	636	12.7	20,001	400.0	15.2	3.79	+ .18 ± .09
425—475	61	837	13.7	27,186	445.7	15.1	3.40	+ .48 ± .07
475—525	41	639	15.6	20,529	500.7	14.8	2.95	+ .43 ± .09
520—580	45	750	16.7	24,846	552.1	18.7	3.39	+ .26 ± .09
550—650	75	1369	18.3	44,972	599.6	29.0	4.84	+ .29 ± .07
650—750	41	680	16.6	28,747	701.1	28.5	4.06	+ .33 ± .09
750—850	47	1138	24.2	37,658	801.2	30.4	3.79	+ .38 ± .08
840—960	40	970	24.3	35,957	898.9	35.6	3.96	+ .40 ± .09
925—1075	48	1374	28.6	47,846	996.8	46.1	4.63	+ .38 ± .08
1000—1200	42	1215	28.9	45,617	1086.1	53.9	4.97	+ .17 ± .10
1050—1350	49	1771	36.1	57,901	1181.7	92.3	7.81	+ .35 ± .08
1150—1450	42	1739	41.4	54,940	1308.1	80.3	6.14	+ .28 ± .10
1200—1600	50	2489	49.8	69,018	1380.4	99.9	7.24	+ .24 ± .09
1300—1700	45	2316	51.5	65,412	1453.6	107.6	7.40	+ .11 ± .10
1400—1800	38	1997	52.6	60,092	1581.4	127.5	8.06	+ .28 ± .10
Hoshiarpur 1907 :								
75—125	50	664	13.3	4984	99.7	14.1	14.12	+ .18 ± .09
175—225	62	681	11.0	12,395	199.9	15.9	7.93	+ .37 ± .07
275—325	51	638	12.5	15,178	297.6	14.5	4.87	+ .14 ± .09
370—430	60	655	10.9	24,135	402.3	19.9	4.94	+ .28 ± .07
450—550	70	1046	14.9	34,631	494.7	28.8	5.82	+ .27 ± .07
550—650	67	1229	18.3	40,143	603.2	31.2	5.17	+ .60 ± .05
650—750	40	720	18.0	27,839	695.4	31.8	4.58	+ .12 ± .11
725—875	64	1203	18.8	51,111	798.6	42.3	5.30	+ .25 ± .08
825—975	57	1234	21.6	51,243	899.0	47.4	5.27	+ .48 ± .07
925—1075	46	1100	23.9	46,029	1000.6	47.3	4.73	+ .34 ± .09
990—1210	42	1243	29.6	45,282	1078.1	56.3	5.23	+ .20 ± .10
1025—1375	55	1440	26.2	64,837	1178.9	117.7	9.98	+ .37 ± .08
1125—1475	43	1212	28.2	56,538	1314.8	96.7	7.36	+ .50 ± .08
1200—1600	48	1358	28.3	67,108	1398.1	97.4	6.96	+ .40 ± .08
1300—1700	49	1588	32.4	71,416	1457.5	107.6	7.38	+ .47 ± .08
1400—1800	40	1285	32.1	63,344	1583.6	122.1	7.71	+ .59 ± .07
Amritsar 1903 :								
100—300	31	845	27.3	6864	221.4	50.1	22.63	+ .09 ± .12
400—500	45	1333	29.6	20,528	456.2	28.2	6.18	+ .02 ± .10

TABLE XIX (*continued*).

Population group	Number of villages	Number of deaths	Mean deaths per village	Population	Mean population of villages	Standard deviation of population	Coefficient of variation	
Amritsar 1903 (<i>continued</i>):								
700—900	72	2849	39.6	57,254	795.2	53.5	6.70	+ .13 ± .08
900—1100	48	2792	58.2	47,709	993.9	64.9	6.53	+ .00 ± .10
1050—1350	56	3254	58.1	66,495	1187.4	90.0	7.58	+ .35 ± .08
1150—1450	47	3182	67.7	60,434	1285.8	85.6	6.66	+ .21 ± .09
1250—1550	48	3354	69.9	67,270	1401.5	85.2	6.08	+ .20 ± .09
1350—1650	43	2916	67.8	64,428	1498.3	78.7	5.25	+ .22 ± .10
1450—1750	43	3094	72.0	67,986	1581.1	91.9	5.81	+ .45 ± .08
1700—2300	45	3776	83.9	86,493	1922.1	146.5	7.62	+ .33 ± .09
1850—3150	43	4133	96.1	96,943	2254.5	374.1	16.59	+ .35 ± .09
2000—4000	40	4750	118.8	108,251	2706.3	572.7	21.16	+ .49 ± .08
Amritsar 1904:								
100—300	42	610	14.5	9536	227.0	48.7	21.44	+ .41 ± .09
400—500	25	477	19.1	11,240	449.6	28.3	6.28	- .38 ± .12
700—900	56	1259	22.5	44,196	789.2	54.0	6.84	+ .11 ± .09
900—1100	41	2051	50.0	40,785	994.8	56.5	5.68	+ .48 ± .08
1050—1350	50	2522	50.4	59,898	1198.0	89.1	7.44	+ .58 ± .06
1150—1450	42	2181	51.9	53,703	1278.6	81.5	6.38	+ .46 ± .08
1250—1550	43	2467	57.4	59,972	1394.7	89.7	6.43	+ .35 ± .09
1350—1650	40	2849	71.2	60,091	1502.3	84.9	5.65	+ .39 ± .09
1450—1750	44	2743	62.3	69,952	1589.8	89.9	5.65	+ .25 ± .10
1700—2300	42	2853	67.9	81,198	1933.3	155.4	8.04	+ .39 ± .09
1900—3100	39	2543	65.2	90,782	2327.7	334.8	14.38	+ .13 ± .11
2000—4000	40	2901	72.5	105,171	2629.3	530.2	20.17	+ .10 ± .11
Amritsar 1905:								
100—300	60	1290	21.5	13,668	227.8	46.9	20.57	+ .20 ± .08
400—500	55	1329	25.2	25,005	454.6	27.9	6.15	+ .42 ± .07
700—900	91	3591	39.5	72,668	798.5	54.4	6.81	+ .33 ± .06
900—1100	60	3301	55.0	59,318	988.6	64.5	6.52	+ .35 ± .08
1100—1300	45	2313	51.4	53,814	1195.9	58.3	4.87	+ .53 ± .07
1200—1400	41	2040	49.8	53,165	1296.7	60.4	4.66	+ .18 ± .10
1250—1550	58	3345	57.7	81,257	1401.0	83.5	5.96	+ .17 ± .09
1350—1650	57	3410	59.8	84,968	1490.7	85.1	5.71	+ .18 ± .09
1450—1750	52	2961	56.9	80,789	1553.6	74.8	4.82	+ .34 ± .08
1700—2300	51	3438	67.4	98,925	1939.7	158.2	8.16	+ .13 ± .09
1900—3100	46	4020	87.4	106,765	2321.0	329.7	14.21	+ .27 ± .09
2000—4000	53	4904	92.5	138,576	2614.6	549.3	21.01	+ .16 ± .09
Amritsar 1907:								
100—300	60	1266	21.1	13,284	221.4	52.5	23.73	+ .31 ± .08
400—500	52	1258	24.2	23,393	449.9	26.9	5.98	+ .30 ± .09
700—900	82	3000	36.6	65,685	801.0	55.1	6.87	+ .29 ± .07
900—1100	57	2136	37.5	56,481	990.9	64.2	6.48	+ .33 ± .08
1100—1300	45	1825	40.6	53,932	1198.5	56.9	4.75	+ .28 ± .09
1150—1450	55	2408	43.8	70,383	1328.0	82.2	6.19	+ .25 ± .09
1250—1550	49	2405	49.1	68,259	1393.0	86.0	6.17	+ .41 ± .08
1350—1650	44	2284	51.9	65,672	1492.5	83.4	5.59	+ .42 ± .08
1450—1750	44	2086	47.4	69,781	1585.9	86.8	5.47	+ .41 ± .08
1700—2300	53	3106	58.6	102,469	1933.4	147.9	7.65	+ .26 ± .09
1900—3100	47	3594	76.5	108,123	2300.5	329.0	14.30	+ .19 ± .09
2000—4000	50	4081	81.6	131,778	2635.6	539.3	20.46	+ .24 ± .09
Gujrat 1907:								
400—500	83	3914	47.2	37,385	450.4	28.0	6.22	+ .35 ± .06
750—850	41	3432	83.7	32,650	796.3	30.7	3.86	+ .21 ± .10
900—1100	52	4777	91.9	52,338	1006.5	62.2	6.18	+ .29 ± .09
1050—1350	47	4945	105.2	54,167	1152.5	70.5	6.12	+ .15 ± .10

TABLE XX.

Mean values of coefficients in Table XIX.

District	Year	Mean r	S.D.	Coeff. of var.
Amritsar	1903	·23	·154	66·96
	1904	·33	·221	66·97
	1905	·27	·113	41·85
	1907	·30	·066	22·00
Hoshiarpur	1902	·38	·062	16·32
	1905	·29	·122	42·07
	1907	·35	·144	41·14
Gujrat	1907	·27	·079	29·26

TABLE XXI.

Correlation between length of exposure and total number of plague deaths for a constant population.

(Villages up to 3000 inhabitants.)

District	Epidemic	No. of villages	Coeff. of correlation
Hoshiarpur	1901—2	447	·25 ± ·03
„	1903—4	844	·24 ± ·02
„	1904—5	950	·24 ± ·02
„	1905—6	284	·29 ± ·04
„	1906—7	875	·25 ± ·02
Gujrat	1903—4	296	·39 ± ·03
„	1904—5	257	·27 ± ·04
„	1906—7	843	·28 ± ·02
Rohtak	1904—5	232	·30 ± ·04
„	1906—7	200	·30 ± ·04
Amritsar	1902—3	456	·19 ± ·03
„	1903—4	412	·34 ± ·03
„	1904—5	643	·29 ± ·02
„	1906—7	560	·28 ± ·03
Mozaffarnagar	1904—5	266	·05 ± ·04
„	1906—7	536	·27 ± ·03

Mean values for different districts.

Hoshiarpur	·25	Amritsar	·28
Gujrat	·30	Mozaffarnagar	·20
Rohtak	·30		

TABLE XXI A.

Amritsar (1902-3). Month of first infection.

Population group	December	January	February	March	April	May	June	Frequency
0—200	1·0	1·0	2·0	3·0	4·0	4·0	—	15·0
200—400	3·0	10·0	5·0	2·0	12·0	14·0	5·0	51·0
400—600	4·0	13·0	14·0	23·0	8·0	17·0	2·0	81·0
600—800	4·0	12·0	12·0	16·0	16·0	12·0	4·0	76·0
800—1000	10·0	6·0	8·0	14·0	12·0	8·0	1·0	59·0
1000—1200	7·0	6·0	9·0	6·0	8·0	3·0	1·0	40·0
1200—1400	7·0	8·0	2·5	7·0	3·0	7·0	—	34·5
1400—1600	1·0	1·0	2·5	6·0	10·0	10·0	—	30·5
1600—1800	4·0	5·0	3·0	6·0	3·0	3·0	—	24·0
1800—2000	6·0	4·0	5·0	1·0	2·0	2·0	—	20·0
2000—2200	—	1·0	2·0	4·0	1·0	4·0	1·0	13·0
2200—2400	1·0	—	—	1·0	2·0	—	—	4·0
2400—2600	—	1·0	—	1·0	1·0	1·0	—	4·0
2600—2800	—	—	—	—	1·0	—	—	1·0
2800—3000	—	1·0	1·0	1·0	—	—	—	3·0
Total	48·0	69·0	66·0	91·0	83·0	85·0	14·0	456·0

TABLE XXI B.

Amritsar (1902-3). Population groups.

Number of deaths	0—200	200—400	400—600	600—800	800—1000	1000—1200	1200—1400	1400—1600	1600—1800	1800—2000	2000—2200	2200—2400	2400—2600	2600—2800	2800—3000	Frequency
1—5	3·0	2·0	4·0	2·0	3·0	2·0	2·0	1·0	—	—	1·0	—	—	—	—	20·0
6—15	2·0	15·0	16·5	14·0	8·0	5·5	2·0	3·0	—	1·5	1·0	—	—	—	—	68·5
15—30	8·0	18·0	25·5	19·0	10·0	5·5	5·0	5·5	4·0	3·0	2·0	—	—	—	—	105·5
30—45	1·0	5·0	18·0	15·0	8·5	3·5	7·5	4·0	2·5	1·5	1·0	—	1·0	—	—	68·5
45—60	1·0	3·0	7·5	15·5	12·0	6·5	5·0	6·0	6·5	·5	1·5	1·0	·5	—	—	66·5
60—75	—	3·5	4·5	4·5	5·5	6·5	3·0	—	—	4·5	·5	—	·5	—	—	33·0
75—90	—	·5	1·5	1·0	5·5	5·0	1·5	2·0	1·5	2·5	1·0	1·0	—	—	—	23·0
90—105	—	1·5	2·5	4·0	4·0	2·0	3·5	5·0	3·5	3·5	4·0	1·0	—	—	—	34·5
105—120	—	·5	—	—	1·0	1·5	—	1·0	2·0	—	—	—	—	—	—	6·0
120—135	—	—	—	1·0	·5	—	—	—	—	—	1·0	—	—	—	2·0	4·5
135—150	—	1·0	1·0	—	—	—	·5	1·0	—	—	—	—	·5	—	1·0	5·0
150—165	—	1·0	—	—	—	—	·5	—	—	—	—	—	·5	1·0	—	3·0
165—180	—	—	—	—	—	·5	·5	—	1·0	—	—	—	1·0	—	—	3·0
180—195	—	—	—	—	—	·5	·5	1·0	1·5	—	—	·5	—	—	—	4·0
195—210	—	—	—	—	—	1·0	1·0	—	·5	—	—	·5	—	—	—	3·0
210—225	—	—	—	—	1·0	—	—	—	—	1·0	—	—	—	—	—	2·0
225—240	—	—	—	—	—	—	1·0	—	—	—	—	—	—	—	—	1·0
240—255	—	—	—	—	—	—	·5	1·0	1·0	—	—	—	—	—	—	2·5
255—270	—	—	—	—	—	—	·5	—	—	1·0	—	—	—	—	—	1·5
270—285	—	—	—	—	—	—	—	—	—	1·0	—	—	—	—	—	1·0
Total	15·0	51·0	81·0	76·0	59·0	40·0	34·5	30·5	24·0	20·0	13·0	4·0	4·0	1·0	3·0	456·0

TABLE XXI c.

Amritsar (1902-3). Month of first infection.

Number of deaths	December	January	February	March	April	May	June	Frequency
1— 5	4.0	5.0	—	2.0	2.0	6.0	1.0	20.0
6— 15	5.5	13.0	8.5	9.0	6.0	18.0	8.5	68.5
15— 30	12.0	17.0	5.5	16.5	22.0	28.0	3.5	104.5
30— 45	3.5	7.0	13.5	15.5	10.5	18.5	1.0	69.5
45— 60	4.0	6.5	11.5	15.5	18.5	10.5	—	66.5
60— 75	2.0	7.5	6.0	8.0	8.5	—	—	32.0
75— 90	4.0	2.0	6.5	5.0	3.5	2.0	—	23.0
90—105	3.5	4.0	11.0	9.5	6.0	.5	—	34.5
105—120	3.5	—	.5	.5	1.0	.5	—	6.0
120—135	—	1.0	—	3.5	1.0	—	—	5.5
135—150	—	1.5	1.0	1.5	—	1.0	—	5.0
150—165	—	1.5	—	.5	1.0	—	—	3.0
165—180	—	2.0	—	.5	.5	—	—	3.0
180—195	—	1.0	—	2.5	.5	—	—	4.0
195—210	2.0	—	—	1.0	—	—	—	3.0
210—225	1.0	—	—	—	1.0	—	—	2.0
225—240	—	—	1.0	—	—	—	—	1.0
240—255	1.5	—	—	—	1.0	—	—	2.5
255—270	.5	—	1.0	—	—	—	—	1.5
270—285	1.0	—	—	—	—	—	—	1.0
Total	48.0	69.0	66.0	91.0	83.0	85.0	14.0	456.0

TABLE XXII.

Average duration of plague in villages infected in different months.

Month of 1st infection	No. of villages first reporting deaths in this month		Average No. of months in which deaths continued to be reported	
	Gujrat 1906-7	Hoshiarpur 1906-7	Gujrat 1906-7	Hoshiarpur 1906-7
December	16	97	5.44	2.96
January	20	122	4.45	2.98
February	56	143	3.91	2.69
March	165	224	3.25	2.18
April	294	192	2.67	1.63
May	223	90	2.04	1.28
June	67	20	1.28	1.10
July	9	4	1.00	1.00

TABLE XXII A.

Death rates of villages reporting deaths in certain sequences.

District and year	Sequence January, February, March			Sequence February, March, April		
	Population	Deaths	Rate	Population	Deaths	Rate
Mozaffarnagar 1907	7135	621	8.70	23,072	1532	6.64
Amritsar 1907	7017	345	4.92	8385	397	4.73
Amritsar 1905	18,177	743	4.09	30,930	1272	4.11
Hoshiarpur 1905	25,424	894	3.52	21,878	658	3.01
Hoshiarpur 1907	15,317	621	4.05	21,757	966	4.44
Total	73,070	3224	4.41	106,022	4825	4.55

TABLE XXIII.

Particulars of epidemics in which over 300 villages were infected in any district.

District and year	Particulars for all villages infected					Infected villages over 3000 population					Infected villages under 3000 population				
	Total population	Number of villages	Total mortality	Death rate per cent.	Average population of villages	Total population	Number of villages	Total mortality	Death rate per cent.	Average population of villages	Total population	Number of villages	Total mortality	Death rate per cent.	Average population of villages
Hoshiarpur :															
1902	404,430	476	11,688	2.89	849.6	63,755	12	864	1.36	5312.9	340,675	464	10,824	3.18	734.2
1904	572,120	856	26,067	4.56	668.4	65,952	12	1425	2.16	5496.0	506,168	844	24,642	4.87	599.7
1905	619,241	952	19,131	3.09	650.5	63,305	12	1052	1.66	5275.4	555,936	940	18,079	3.25	591.4
1907	598,384	892	15,707	2.63	670.8	68,079	13	962	1.41	5236.8	530,305	879	14,745	2.78	603.3
Amritsar :															
1903	537,611	479	26,825	4.99	1122.4	104,317	25.5	3839	3.68	4090.8	433,294	453.5	22,986	5.30	955.4
1904	509,119	429	18,372	3.61	1186.8	112,456	26.0	3184	2.83	4325.2	396,663	403.0	15,188	3.83	984.3
1905	683,719	643	29,646	4.34	1063.3	130,630	31.5	4263	3.26	4147.0	553,089	611.5	25,383	4.59	904.5
1907	633,670	590	23,642	3.73	1074.0	120,547	28.5	3010	2.50	4229.7	513,123	561.5	20,632	4.02	913.8
Gujrat :															
1904	277,151	305	23,785	8.58	908.7	69,671	9	849	1.22	7741.2	207,480	296	22,936	11.05	700.9
1907	560,579	853	51,000	9.10	657.2	68,231	10	2718	3.98	6823.1	492,348	843	48,282	9.81	584.0
Mozaffarnagar :															
1905	545,689	316	16,246	2.98	1726.9	247,026	43	6961	2.82	5744.8	298,663	273	9285	3.11	1094.0
1907	753,403	590	49,909	6.62	1277.0	264,202	49	16,365	6.19	5391.9	489,201	541	33,544	6.86	904.3

TABLE XXIV.

Particulars of epidemics in which less than 300 villages were infected in any district.

District and year	Particulars for all villages infected					Infected villages over 300 population					Infected villages under 300 population				
	Total popu- lation	Number of villages	Total mor- tality	Death rate per cent.	Average population of villages	Total popu- lation	Number of villages	Total mor- tality	Death rate per cent.	Average population of villages	Total popu- lation	Number of villages	Total mor- tality	Death rate per cent.	Average population of villages
Hoshiarpur :															
1898	17,438	16	439	2.52	1089.9	5803	1	87	1.50	5803.0	11,635	15	352	3.03	775.7
1899	3701	4	9	.24	925.3	—	—	—	—	—	3701	4	9	.24	925.3
1900	9636	11	50	.52	876.0	—	—	—	—	—	9636	11	50	.52	876.0
1901	42,790	45	444	1.04	950.9	9030	2	112	1.24	4515.0	33,760	43	332	.98	785.1
1903?	No data	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1906	228,728	293	4125	1.80	780.6	52,038	9	612	1.18	5782.0	176,690	284	3513	1.99	622.1
Gujrat :															
1902	72,200	38	551	.76	1900.0	37,875	3	14	.04	12,625.0	34,325	35	537	1.56	980.7
1903	87,724	58	1794	2.05	1512.5	50,902	6	673	1.32	8483.7	36,822	52	1121	3.04	708.1
1905	231,535	262	9782	4.22	883.7	46,619	5	775	1.66	9323.8	184,916	257	9007	4.87	719.5
1906	120,219	60	1207	1.00	2000.7	81,328	8	40	.05	10,166.0	38,891	52	1167	3.00	747.9
Mozaffarnagar :															
1902	25,581	2	4	.02	12,790.5	23,444	1	1	.00	23,444.0	2137	1	3	.14	2137.0
1903	73,944	23	782	1.05	3215.0	50,509	5	257	.51	10,101.8	23,435	18	525	2.24	1301.9
1904	257,455	128	5486	2.13	2011.4	131,227	20	2371	1.81	6561.4	126,228	108	3115	2.47	1168.8
1906	174,409	68	3077	1.76	2564.8	103,924	14	1526	1.47	7423.1	70,485	54	1551	2.20	1305.3
Rohtak :															
1904	147,918	56	2990	2.02	2641.4	108,513	17	1996	1.84	6383.1	39,105	39	994	2.52	1010.4
1905	491,746	284	28,802	5.86	1731.5	229,747	46	12,792	5.57	4994.5	261,999	238	16,010	6.11	1100.8
1906	121,872	31	1948	1.60	3931.4	97,761	16	1275	1.30	6110.1	24,111	15	673	2.79	1607.4
1907	469,355	247	31,330	6.68	1900.2	222,249	44	14,619	6.58	5096.6	247,106	203	16,711	6.76	1217.3
Amritsar :															
1902	97,154	58	1957	2.01	1675.1	34,514	7	651	1.89	4930.6	62,640	51	1306	2.08	1228.2
1906	339,770	272	6008	1.77	1249.2	90,985	21.5	1217	1.34	4231.9	248,785	250.5	4791	1.93	993.2

TABLE XXV.

Plague experience in towns of over 13,500 inhabitants.

Name of town	Population	Number of deaths	Mortality per cent.	Month of 1st infection	Number of months epidemic continued
Rohtak Town :					
1904	20,024	13	·065	February	4
1905	20,024	888	4·435	Oct. '04	8
1906	20,024	3	·015	April	2
1907	20,024	464	2·317	February	5
Amritsar City :					
1902	162,429	82	·051	February	Continuously infected.
1903	162,429	460	·283	Continuous	
1904	162,429	1103	·679	January	7
1905	162,429	1073	·661	January	20
1906	162,429	1903	1·172	Continuous	
1907	162,429	1101	·678	January	7
Mozaffarnagar :					
1902	23,444	1	·004	January	1
1903	23,444	25	·107	February	5
1904	23,444	331	1·412	Oct. '03	7
1905	23,444	249	1·062	Nov. '04	7
1906	23,444	112	·478	March	4
* 1907	23,444	490	2·090	Nov. '06	7
Kairana (Mozaffarnagar District) :					
1905	19,304	94	·487	January	6
1906	19,304	2	·010	April	1
1907	19,304	2136	11·065	February	5
Gujrat :					
1902	19,708	11	·056	May	2
1903	19,708	281	1·426	Aug. '02	12
1904	19,708	89	·452	Oct. '03	10
1905	19,708	183	·929	Oct. '04	9
1906	19,708	18	·091	April	2
1907	19,708	237	1·203	Oct. '06	10

* One further case occurred here in Sept. 1907, three months after epidemic ceased.

TABLE XXVI.

HOSHIARPUR 1907.

Infected villages having less than 1000 population, showing month of 1st infection.

Month of 1st infection	Population	Number of villages	Number of deaths	Mortality per cent.	Average population of villages
Dec. '06	32,914	74	1277	3·88	444·8
January	36,667	87	1529	4·17	421·5
February	53,767	117	2204	4·10	459·5
March	71,666	185	2681	3·74	387·4
April	65,994	167	1585	2·40	395·2
May	32,643	85	524	1·61	384·0
June	7486	16	86	1·15	467·9
July	1535	4	14	·91	383·8
Total	302,672	735	9900	3·27	411·8

Infected villages having population over 1000 and under 3000, showing month of 1st infection.

Dec. '06	40,689	25	1198	2·94	1627·6
January	43,073	29	1054	2·45	1485·3
February	28,723	19	865	3·01	1511·7
March	62,258	39	1256	2·02	1596·4
April	40,908	24	663	1·62	1704·5
May	7048	5	45	·64	1409·6
June	3945	2	13	·33	1972·5
July	1213	1	1	·08	1213·0
Total	227,857	144	5095	2·24	1582·3

TABLE XXVII.

AMRITSAR 1907.

Infected villages having less than 1000 population, with month of 1st infection.

Month of 1st infection	Population	Number of villages	Number of deaths	Mortality per cent.	Average population of villages
Dec. '06	13,900	22	1011	7·27	631·8
January	9557	17	496	5·19	562·2
February	12,569	23	715	5·69	546·5
March	41,537	73	2837	6·83	569·0
April	66,540	122	3535	5·31	545·4
May	52,056	95	1793	3·44	548·0
June	9087	21	173	1·90	432·7
Total	205,246	373	10,560	5·15	550·3

Villages with population from 1000—3000, and month of 1st infection.

Dec. '06	26,407	17	1124	4·26	1553·4
January	12,699	9	482	3·80	1411·0
February	40,448	25	1481	3·66	1617·9
March	78,808	46	2816	3·57	1713·2
April	102,760	65	3406	3·31	1580·9
May	48,149	33	974	2·02	1459·1
June	8878	6	87	·98	1479·7
Total	318,149	201	10,370	3·26	1582·8

Village Plague

TABLE XXVIII.

MOZAFFARNAGAR 1907.

Infected villages having less than 1000 population, showing month of 1st infection.

Month of 1st infection	Population	Number of villages	Number of deaths	Mortality per cent.	Average population of villages
Dec. '06	10,292	16	375	3·64	643·3
January	9839	16	887	9·02	614·9
February	20,686	39	2315	11·19	530·4
March	42,767	77	4365	10·21	555·4
April	58,164	118	4026	6·92	492·9
May	35,079	68	1176	3·35	515·9
June	3304	7	68	2·06	472·0
Total	180,131	341	13,212	7·33	528·2

Infected villages with population from 1000 to 3000, showing month of 1st infection.

Dec. '06	32,425	18	1727	5·33	1801·4
January	41,076	25	3594	8·75	1643·0
February	41,792	27	3574	8·55	1547·9
March	77,593	48	6201	7·99	1616·5
April	71,259	47	4123	5·79	1516·1
May	38,740	26	631	1·63	1490·0
June	4102	3	56	1·37	1367·3
Total	306,987	194	19,906	6·48	1582·4

TABLE XXIX.

GUJRAT 1907.

Infected villages with population up to 1000, showing month of 1st infection.

Month of 1st infection	Population	Number of villages	Number of deaths	Mortality per cent.	Average population of villages
Dec. '06	2494	5	264	10·59	498·8
January	8509	16	1252	14·71	531·8
February	18,364	43	2894	15·76	427·1
March	64,209	132	9144	14·24	486·4
April	103,341	243	12,726	12·31	425·3
May	76,499	203	4350	5·69	376·8
June	20,552	64	509	2·48	321·1
July	2402	8	34	1·42	300·3
Total	296,370	714	31,173	10·52	405·1

Infected villages from 1000 to 3000 population, with month of 1st infection.

Dec. '06	14,002	9	887	6·33	1555·8
January	7315	4	657	8·98	1828·8
February	19,353	12	2806	14·50	1612·8
March	47,987	31	5275	10·99	1548·0
April	73,958	48	6780	9·17	1540·8
May	26,498	21	634	2·39	1261·8
June	5462	3	54	·99	1820·7
July	1403	1	5	·36	1403·0
Total	195,978	129	17,098	8·72	1519·2

TABLE XXX.

ROHTAK 1907.

*Infected villages having less than 1000 population, showing
month of 1st infection.*

Month of 1st infection	Population	Number of villages	Number of deaths	Mortality per cent.	Average popula- tion of villages
Dec. '06	—	—	—	—	—
January	1082	2	53	4·90	541·0
February	6428	12	706	10·98	535·7
March	12,592	21	1050	8·34	599·6
April	17,688	31	1077	6·09	570·6
May	13,565	20	318	2·34	678·3
June	3091	5	20	·65	618·2
Total	54,446	91	3224	5·92	598·3

*Infected villages with populations from 1000 to 3000, showing
month of 1st infection.*

Dec. '06	10,257	5	631	6·15	2051·4
January	4707	4	416	8·84	1176·8
February	7089	5	628	8·86	1417·8
March	85,310	47	8011	9·39	1815·1
April	64,589	38	3478	5·38	1699·7
May	19,348	12	300	1·55	1612·3
June	1360	1	23	1·69	1360·0
Total	192,660	112	13,487	7·00	1720·2

TABLE XXXI.

Comparison of mortality in villages with population of under 300 with the mortality in those having a population of 300 or over in certain districts and epidemics.

Population under 300.						
District and year	Infected first in month of	Total population	Number of villages	Total number of deaths	Mortality per cent.	Average population of villages
Gujrat 1907	April	17,627	92	2673	15·16	191·60
	May	16,445	88	1178	7·16	186·88
Hoshiarpur 1904	April	15,967	88	1429	8·95	181·44
	May	16,889	95	1013	6·00	177·78
Hoshiarpur 1907	March	15,099	82	892	5·91	184·13
	April	12,436	67	567	4·56	185·61
Population 300 or over.						
Gujrat 1907	April	173,047	202	17,071	9·86	856·67
	May	86,552	136	3806	4·40	636·41
Hoshiarpur 1904	April	120,786	161	5618	4·65	750·22
	May	110,711	148	3582	3·24	748·05
Hoshiarpur 1907	March	118,825	162	3045	2·56	733·49
	April	100,951	125	1681	1·67	807·61
All infected villages.						
Gujrat 1907	April	190,674	294	19,744	10·36	648·55
	May	102,997	224	4984	4·84	459·80
Hoshiarpur 1904	April	136,753	249	7047	5·15	549·10
	May	127,600	243	4595	3·60	525·10
Hoshiarpur 1907	March	133,924	224	3937	2·94	597·88
	April	113,387	192	2248	1·98	590·56

TABLE XXXII.

ROHTAK 1907.

Tehsil	Villages with population below 1000			Villages with population between 1000—3000		
	Total population	Deaths	Percentage	Total population	Deaths	Percentage
Rohtak	13,794	774	5·6	39,529	1547	3·9
Gohannah	11,434	686	6·0	69,668	5072	7·3
Sampla	20,052	1374	6·9	73,518	6665	9·1
Jhajjar	9166	390	4·3	9945	203	2·0

TABLE XXXIII.

Proportions of villages in different groups infected in 1907.*

District	Mean population of villages 0—1000	Mean population of villages 1000—3000	Percentage No. of villages 0—1000 infected 1906—7	Percentage No. of villages 1000—3000 infected 1906—7	Ratio of percentage infections for the two groups	Ratio of mean populations for the two groups
Rohtak	458·5	1655·7	30·4	73·7	2·4	3·6
Amritsar	457·8	1539·3	47·7	81·7	1·7	3·4
Mozaffarnagar	413·4	1584·6	48·8	86·2	1·8	3·8
Hoshiarpur	334·5	1525·0	39·3	67·3	1·7	4·6

* Gujrat not included owing to uncertainties respecting populations of non-infected villages.

TABLE XXXIV.

Comparison of mortality rates including non-infected villages, 1907.

Villages having less than 1000 population.

District	Population	Number of villages	Number of deaths	Mortality per cent.	Average population of villages
Rohtak	137,080	299	3224	2·35	458·5
Amritsar	357,961	782	10,560	2·95	457·8
Mozaffarnagar	288,964	699	13,212	4·57	413·4
Hoshiarpur	625,822	1871	9900	1·58	334·5

Villages having population of 1000—3000.

Rohtak	251,662	152	13,487	5·36	1655·7
Amritsar	378,657	246	10,370	2·74	1539·3
Mozaffarnagar	356,528	225	19,906	5·58	1584·6
Hoshiarpur	326,359	214	5095	1·56	1525·0

TABLE XXXV.

GUJRAT 1907.

Villages infected in 2 months :

	Population	Deaths	Number of villages	D.R.	Ratio	Average population
Up to 1000	81,009	8476	224	10·46	2·24	361·6
1000—3000	31,925	1495	24	4·68	—	1330·2

Villages infected in 3 months :

Up to 1000	107,763	12,424	232	11·53	1·16	464·5
1000—3000	72,732	7238	51	9·95	—	1426·1

Villages infected in 4 months :

Up to 1000	49,097	6316	93	12·86	1·17	427·9
1000—3000	43,800	4811	27	10·98	—	1622·2

HOSHIARPUR 1907.

Villages infected 2 months :

Up to 1000	64,553	2158	149	3·34	1·87	433·2
1000—3000	39,269	704	26	1·79	—	1510·3

Villages infected 3 months :

Up to 1000	59,744	3070	132	5·14	2·32	352·6
1000—3000	46,229	1019	29	2·22	—	1594·1

Villages infected 4 months :

Up to 1000	27,897	1642	58	5·89	1·86	481·0
1000—3000	42,447	1341	28	3·16	—	1516·0

TABLE XXXVI.

AMRITSAR 1907.

Villages infected during 2 months :						
	Population	Deaths	Number of villages	D.R.	Ratio	Average population
Up to 1000	78,136	2998	156	3·84	2·48	500·9
1000—3000	62,717	971	45	1·55	—	1393·7
Villages infected during 3 months :						
Up to 1000	65,628	3735	113	5·69	1·64	580·8
1000—3000	115,058	3980	71	3·46	—	1620·5
Villages infected during 4 months :						
Up to 1000	66,655	2478	42	9·30	2·27	634·6
1000—3000	61,601	2527	38	4·10	—	1621·1

TABLE XXXVII.

MOZAFFARNAGAR 1907.

Villages infected 2 months :						
	Population	Deaths	Number of villages	D.R.	Ratio	Average population
Up to 1000	60,531	3956	116	6·54	1·53	521·8
1000—3000	58,216	2486	39	4·27	—	1492·7
Villages infected 3 months :						
Up to 1000	48,425	5527	82	11·41	1·58	590·5
1000—3000	97,987	7069	65	7·21	—	1507·5
Villages infected 4 months :						
Up to 1000	15,923	1830	28	11·49	1·31	568·7
1000—3000	66,720	5840	39	8·75	—	1710·8

TABLE XXXVIII.

ROHTAK 1907.

Villages infected 2 months :						
	Population	Deaths	Number of villages	D.R.	Ratio	Average population
Up to 1000	21,834	1212	34	5·55	1·67	642·2
1000—3000	41,819	1394	25	3·33	—	1672·8
Villages infected 3 months :						
Up to 1000	16,781	1228	28	7·32	·83	599·3
1000—3000	79,156	6984	48	8·82	—	1649·1
Villages infected 4 months :						
Up to 1000	4279	483	6	11·29	1·35	713·2
1000—3000	45,436	3800	23	8·36	—	1975·5

TABLE XXXIX.

General comparison of climatic conditions in the different districts, five months January—May (inclusive).

Year	Amritsar				Rohtak				Gujrat				Hoshiarpur				Mozaffarnagar			
	Mean mortality per cent. 3000 population Villages less than	Average rainfall	Average number of wet days	Mean mortality per cent. 3000 population Villages less than	Mean mortality per cent. 3000 population Villages less than	Average rainfall	Average number of wet days	Mean mortality per cent. 3000 population Villages less than	Mean mortality per cent. 3000 population Villages less than	Average rainfall	Average number of wet days	Mean mortality per cent. 3000 population Villages less than	Mean mortality per cent. 3000 population Villages less than	Average rainfall	Average number of wet days	Mean mortality per cent. 3000 population Villages less than	Mean mortality per cent. 3000 population Villages less than	Average rainfall	Average number of wet days	
1902	2.08	1.511	4.125	—	1.342	2.20	1.56	2.42	7.67	3.18	2.77	7.80	.14	.648	1.50	1.50	2.24	2.47	4.300	9.67
1903	5.03	4.583	6.625	—	.528	2.00	3.04	4.55	12.33	No data	13.144	11.00	2.24	1.095	3.17	2.24	2.47	4.300	9.67	
1904	3.83	6.430	13.50	2.52	2.222	5.80	11.05	9.47	15.33	4.87	7.036	15.00	2.47	4.300	9.67	2.47	4.300	9.67	9.67	
1905	4.59	4.429	11.875	6.11	3.10	7.00	4.87	6.96	13.33	3.25	8.37	16.40	3.11	3.720	8.67	3.11	3.720	8.67	8.67	
1906	1.03	6.264	12.875	2.79	3.022	4.60	3.00	8.643	14.00	1.99	6.364	14.40	2.20	5.203	9.50	2.20	5.203	9.50	9.50	
1907	4.02	7.429	17.00	6.85	4.144	8.00	9.81	14.803	22.00	2.78	15.022	22.40	6.86	6.870	12.67	6.86	6.870	12.67	12.67	

Comparison of mean daily temperature of Lahore with plague mortality in Amritsar district.

January							February							March							April							May						
Year	Mean mortality of villages where epidemic com- menced in Jan.	Mean daily tem- perature	Mean daily range	Mean mortality of villages where epidemic com- menced in Feb.	Mean daily tem- perature	Mean daily range	Mean mortality of villages where epidemic com- menced in Mar.	Mean daily tem- perature	Mean daily range	Mean mortality of villages where epidemic com- menced in April	Mean daily tem- perature	Mean daily range	Mean mortality of villages where epidemic com- menced in May	Mean daily tem- perature	Mean daily range	Mean mortality of villages where epidemic com- menced in May	Mean daily tem- perature	Mean daily range	Mean mortality of villages where epidemic com- menced in May	Mean daily tem- perature														
1903	5.26	55.1	27.7	6.00	59.9	27.5	5.79	66.8	24.6	5.19	76.9	28.6	2.84	87.2	28.9	2.84	87.2	28.9	28.9															
1904	3.81	53.1	22.3	6.15	60.4	30.0	5.47	67.1	19.3	3.56	81.1	30.5	1.32	91.0	28.4	1.32	91.0	28.4	28.4															
1905	4.71	51.6	18.6	5.04	50.1	21.2	4.67	63.6	20.9	4.05	78.0	28.9	2.23	92.7	29.4	2.23	92.7	29.4	29.4															
1906	2.89	53.1	28.9	2.60	56.8	19.1	2.58	64.9	21.7	2.41	78.5	28.7	.721	92.9	31.2	.721	92.9	31.2	31.2															
1907	4.22	58.7	26.3	4.23	56.5	18.8	4.33	64.2	22.8	3.62	77.2	24.1	2.79	87.5	29.3	2.79	87.5	29.3	29.3															

Mean daily temperature, five months January—May (inclusive), Lahore

1903	69.18	1904	70.54	1905	67.20	1906	69.24	1907	68.82
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" " range

27.46	26.10	23.80	25.92	24.26
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Mean percentage mortality for epidemics in Amritsar district

5.03	3.83	4.59	1.93	4.02
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TABLE XL.

*Amritsar City. Monthly deaths and meteorological conditions.
Feb.—July, 1904—1907.*

Month and Year	Number of deaths	Proportion of deaths in the whole 6 months	Rainfall	Number of days upon which rain fell	Mean temperature for the month (Lahore record)
February 1904	0	0	·10	1	60·4°
„ 1905	237	·311	1·05	5	50·1
„ 1906	146	·081	2·90	5	56·8
„ 1907	30	·027	2·50	7	56·5
March 1904	36	·047	4·13	10	67·1°
„ 1905	169	·221	·37	2	63·6
„ 1906	333	·184	1·75	5	64·9
„ 1907	125	·114	1·95	5	64·2
April 1904	359	·471	·06	0	81·1°
„ 1905	127	·166	0	0	78·0
„ 1906	656	·363	·45	2	78·5
„ 1907	417	·382	1·20	3	77·2
May 1904	237	·311	·25	2	91·0°
„ 1905	170	·223	0	0	92·7
„ 1906	581	·321	·20	1	92·9
„ 1907	408	·374	·53	2	87·5
June 1904	109	·143	1·80	2	95·7°
„ 1905	48	·063	·25	1	96·4
„ 1906	83	·046	1·90	3	93·6
„ 1907	111	·102	·60	2	92·0
July 1904	22	·029	·10	1	93·0°
„ 1905	12	·016	3·55	6	92·1
„ 1906	9	·005	3·70	5	93·2
„ 1907	1	·001	1·80	4	93·0

TABLE XLI. *Hoshiarpur District 1902.*

HOSHIARPUR TEHSIL.					DASUYAH TEHSIL.						
Month of infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popm. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popm. of villages
Dec. '01	21,987	1049	4.77	18	1221.5	Dec. '01	1133	1	.09	1	1133.0
January	29,118	855	2.94	16	1819.9	January	861	2	.23	2	430.5
February	30,473	1053	3.46	33	923.4	February	1294	23	1.78	1	1294.0
March	23,224	409	1.76	26	893.2	March	6534	138	2.11	7	933.4
April	22,317	363	1.63	24	929.9	April	10,017	341	3.40	4	2504.3
May	10,040	140	1.39	19	528.4	May	4040	9	.22	2	2020.0
June	905	3	.33	2	452.5	June	—	—	—	—	—
July	2219	36	1.62	4	554.7	July	2543	15	.59	2	1271.5
Total	140,283	3908	2.79	142	987.9	Total	26,422	529	2.00	19	1390.6
GARSHANKAR TEHSIL.					UNA TEHSIL.						
Dec. '01	74,722	3233	4.33	93	803.5	Dec. '01	6550	260	3.97	4	1637.5
January	51,784	1832	3.54	69	750.5	January	2537	31	1.22	2	1268.5
February	30,480	979	3.21	58	525.5	February	9569	287	3.00	6	1594.8
March	24,853	355	1.43	35	710.1	March	12,862	55	.43	11	1169.3
April	11,959	176	1.47	20	597.9	April	3625	16	.44	4	906.3
May	7040	23	.33	10	704.0	May	1744	4	.23	3	581.3
June	—	—	—	—	—	June	—	—	—	—	—
July	—	—	—	—	—	July	—	—	—	—	—
Total	200,838	6598	3.29	285	704.7	Total	36,887	653	1.77	30	1229.6

TABLE XLII. *Hoshiarpur District 1904.*

HOSHARPUR TEHSIL.					DASUYAH TEHSIL.						
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '03	—	—	—	—	—	Dec. '03	—	—	—	—	—
January	21,760	828	3·81	13	1673·8	January	20,963	1097	5·23	31	676·2
February	32,861	1652	5·03	20	1643·0	February	38,483	2722	7·07	43	895·0
March	30,053	1716	5·71	25	1202·1	March	23,612	1317	5·58	43	549·1
April	25,206	1466	5·82	50	504·1	April	42,048	2474	5·88	99	424·7
May	60,904	2466	4·05	101	603·0	May	26,265	971	3·70	64	410·4
June	14,147	202	1·43	32	442·1	June	4384	88	2·01	10	438·4
July	5028	216	4·30	8	628·5	July	414	2	·48	2	207·0
Total	189,959	8546	4·50	249	702·9	Total	156,169	8671	5·55	292	534·8
GARSHPUR TEHSIL.					UNA TEHSIL.						
Dec. '03	—	—	—	—	—	Dec. '03	—	—	—	—	—
January	18,190	530	2·91	15	1212·7	January	1987	51	2·57	2	993·5
February	23,414	1410	6·02	27	867·2	February	1433	21	1·47	2	716·5
March	45,497	2363	5·19	64	710·9	March	11,120	49	·44	4	2780·0
April	62,050	3041	4·90	91	681·9	April	7449	66	·89	9	827·7
May	31,132	986	3·17	65	479·0	May	9299	172	1·85	13	715·3
June	11,645	156	1·34	20	582·3	June	—	—	—	—	—
July	495	2	·40	1	495·0	July	2281	3	·13	2	1140·5
Total	192,423	8488	4·41	283	679·9	Total	33,569	362	1·08	32	1049·0
TOTAL HOSHARPUR DISTRICT 1904.					TOTAL HOSHARPUR DISTRICT 1904.						
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '03	—	—	—	—	—	Dec. '03	—	—	—	—	—
January	62,900	2506	3·98	61	1031·1	January	61	1031·1	—	—	—
February	96,191	5805	6·04	92	1045·6	February	92	1045·6	—	—	—
March	110,282	5445	4·94	136	810·9	March	136	810·9	—	—	—
April	136,753	7047	5·15	249	549·1	April	249	549·1	—	—	—
May	127,600	4595	3·60	243	525·1	May	243	525·1	—	—	—
June	30,176	446	1·48	62	486·7	June	62	486·7	—	—	—
July	8218	223	2·71	13	632·2	July	13	632·2	—	—	—
Total	572,120	26,067	4·56	856	668·4	Total	856	668·4	—	—	—

TABLE XLIII. *Hoshiarpur District 1905.*

HOSHIAHPUR TEHSIL.						DASUYAN TEHSIL.					
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '04	46,628	2299	4.93	64	728.6	Dec. '04	12,742	611	4.80	13	980.2
January	76,700	2532	3.30	71	1080.3	January	42,372	1333	3.15	65	651.9
February	24,660	541	2.19	31	725.3	February	18,550	484	2.61	24	772.9
March	22,719	615	2.71	42	540.9	March	15,957	443	2.78	24	664.9
April	22,368	559	2.50	47	475.9	April	16,477	481	2.92	42	392.3
May	15,479	245	1.58	28	552.8	May	15,399	360	2.34	33	466.6
June	8189	129	1.58	19	431.0	June	13,595	204	1.50	28	485.5
July	1566	4	.26	3	522.0	July	1781	24	1.35	7	254.4
Total	218,309	6924	3.17	308	708.8	Total	136,873	3940	2.88	236	580.0

GARSHANKAR TEHSIL.						UNA TEHSIL.					
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '04	616	12	1.95	3	205.3	Dec. '04	14,892	593	3.98	21	709.1
January	58,570	2192	3.74	75	780.9	January	17,128	439	2.56	19	901.5
February	31,535	1169	3.71	43	733.4	February	14,608	228	1.55	19	772.0
March	35,327	1403	3.97	62	569.8	March	9747	135	1.39	18	541.5
April	50,748	1394	2.75	76	667.7	April	12,115	250	2.06	29	417.8
May	16,969	356	2.10	32	530.3	May	3161	47	1.49	9	351.2
June	7558	102	1.35	20	377.9	June	1185	1	.08	1	1185.0
July	1356	13	.96	3	452.0	July	2841	48	1.69	3	947.0
Total	202,679	6641	3.28	314	645.5	Total	75,737	1741	2.30	119	636.4

TOTAL HOSHIAHPUR DISTRICT 1905.					
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '04	74,878	3515	4.69	101	741.4
January	194,770	6496	3.34	230	846.8
February	89,413	2422	2.71	120	745.1
March	83,750	2596	3.10	146	573.6
April	101,708	2684	2.64	194	524.6
May	51,008	1008	1.98	102	500.1
June	30,527	436	1.43	68	448.9
July	7544	89	1.18	16	471.5
Total	633,598	19,246	3.04	977	648.5

TABLE XLIV. *Hoshiarpur District 1907.*

HOSHIARPUR TEHSIL.					DASTYAH TEHSIL.				
Month of 1st infection	Population	No. of deaths	Mortality per cent.	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	Average popn. of villages
Dec. '06	21,134	639	3.02	845.4	Dec. '06	23,510	875	3.72	587.8
January	46,257	1233	2.67	1321.6	January	36,717	976	2.66	874.2
February	24,215	894	3.69	563.1	February	18,093	736	4.07	489.0
March	44,635	863	1.93	666.2	March	25,716	1142	4.44	459.2
April	37,029	527	1.42	673.3	April	27,765	750	2.70	555.3
May	16,155	172	1.06	489.5	May	4963	105	2.12	275.7
June	12,469	82	.66	1246.9	June	2094	11	.53	349.0
July	619	2	.32	309.5	July	1213	1	.08	1213.0
Total	202,513	4412	2.18	750.0	Total	140,071	4596	3.28	560.3
GARSTANKAR TEHSIL.					UNA TEHSIL.				
Month of 1st infection	Population	No. of deaths	Mortality per cent.	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	Average popn. of villages
Dec. '06	26,304	815	3.10	848.5	Dec. '06	2443	146	5.98	814.3
January	35,858	1096	3.07	833.9	January	976	41	4.20	325.3
February	42,704	1221	2.86	805.7	February	11,972	218	1.82	1945.3
March	46,321	1382	2.98	579.0	March	17,252	550	3.19	821.5
April	32,102	661	2.06	509.6	April	16,491	310	1.88	687.1
May	7744	182	2.35	484.0	May	10,829	110	1.02	470.8
June	1639	3	.18	819.5	June	2261	12	.53	1330.5
July	497	11	2.21	497.0	July	419	1	.24	419.0
Total	193,169	5371	2.78	668.4	Total	62,643	1388	2.22	754.7
TOTAL HOSHIARPUR DISTRICT 1907.					TOTAL HOSHIARPUR DISTRICT 1907.				
Month of 1st infection	Population	No. of deaths	Mortality per cent.	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	Average popn. of villages
Dec. '06	73,391	2475	3.37	99	Dec. '06	73,391	2475	3.37	99
January	119,808	3346	2.79	123	January	119,808	3346	2.79	123
February	96,984	3069	3.16	139	February	96,984	3069	3.16	139
March	133,924	3937	2.94	224	March	133,924	3937	2.94	224
April	113,387	2248	1.98	192	April	113,387	2248	1.98	192
May	39,691	569	1.43	90	May	39,691	569	1.43	90
June	18,463	108	.58	20	June	18,463	108	.58	20
July	2748	15	.55	5	July	2748	15	.55	5
Total	598,396	15,767	2.63	892	Total	598,396	15,767	2.63	892

TABLE XLVI. *Amritsar* 1904.

AMRITSAR TEHSIL.						TARN-TARN TEHSIL.					
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '03	1700	31	1.82	1	1700.0	Dec. '03	—	—	—	—	—
January	26,560	1053	3.96	13	2043.1	January	15,902	686	4.31	6	2650.3
February	17,224	1115	6.47	12	1435.3	February	8514	580	6.81	7	1216.3
March	47,491	2897	6.10	40	1187.3	March	31,521	1597	5.07	20	1576.1
April	84,713	3080	3.64	93	910.9	April	102,974	3998	3.88	80	1287.2
May	37,497	547	1.46	37	1013.4	May	38,789	418	1.08	44	881.6
June	1808	9	.50	3	602.7	June	71	1	1.41	1	71.0
Total	216,993	8732	4.02	199	1090.4	Total	197,771	7280	3.68	158	1251.7
ANALA TEHSIL.						TOTAL AMRITSAR DISTRICT 1904.					
Dec. '03	2179	25	1.15	1	2179.0	Dec. '03	3879	56	1.44	2	1939.5
January	2558	4	.16	1	2558.0	January	45,020	1743	3.87	20	2250.0
February	8469	408	4.82	4	2117.3	February	34,207	2103	6.15	23	1487.3
March	10,284	392	3.81	10	1028.4	March	89,296	4886	5.47	70	1275.7
April	36,239	902	2.49	34	1065.9	April	223,926	7980	3.56	207	1081.8
May	15,851	252	1.59	19	834.3	May	92,137	1217	1.32	100	921.4
June	—	—	—	—	—	June	1879	10	.53	4	469.8
Total	75,580	1983	2.62	69	1095.4	Total	490,344	17,995	3.67	426	1151.0

TABLE XLVII. *Amritsar* 1905.

AMRITSAR TEHSIL.						TARN-TARN TEHSIL.					
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '04	49,893	2524	5.06	38	1313.0	Dec. '04	47,944	2994	6.24	28	1712.4
January	75,278	3489	4.63	71	1060.3	January	34,212	1857	5.43	37	924.7
February	46,119	2090	4.53	47	981.3	February	50,381	2747	5.45	40	1259.5
March	52,869	2161	4.09	55	961.3	March	64,179	3769	5.87	56	1146.1
April	35,318	1055	2.99	36	981.1	April	55,319	2132	3.85	55	1005.8
May	11,658	211	1.81	14	832.7	May	26,025	584	2.24	31	839.5
June	—	—	—	—	—	June	—	—	—	—	—
Total	271,135	11,530	4.25	261	1038.8	Total	278,060	14,083	5.06	247	1125.7
AJNALA TEHSIL.						TOTAL AMRITSAR DISTRICT 1905.					
Dec. '04	12,531	319	2.55	9	1392.3	Dec. '04	110,368	5837	5.29	75	1471.5
January	10,896	351	3.22	10	1089.6	January	120,386	5697	4.73	118	1020.2
February	5658	316	5.59	8	707.3	February	102,158	5153	5.04	95	1075.3
March	23,362	738	3.16	18	1297.9	March	140,410	6668	4.75	129	1088.4
April	28,596	1474	5.15	35	817.0	April	119,233	4661	3.91	126	946.3
May	39,715	925	2.33	49	810.5	May	77,398	1720	2.22	94	823.4
June	2855	38	1.33	6	475.8	June	2855	38	1.33	6	475.8
Total	123,613	4161	3.37	135	915.7	Total	672,808	29,774	4.43	643	1046.4

TABLE XLVIII. *Amritsar* 1906.

AMRITSAR TEHSIL.						TARN-TARN TEHSIL.					
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '05	2463	68	2.76	2	1231.5	Dec. '05	—	—	—	—	—
January	8710	211	2.42	7	1244.3	January	9987	330	3.30	5	1997.4
February	12,299	353	2.87	7	1757.0	February	2208	25	1.13	1	2208.0
March	40,737	1335	3.28	25	1629.5	March	17,674	213	1.21	11	1606.7
April	66,272	1809	2.73	72	920.4	April	28,637	449	1.57	22	1301.7
May	51,000	407	.80	53	962.3	May	33,609	158	.47	27	1244.8
June	11,359	69	.61	9	1262.1	June	6694	6	.09	5	1338.8
Total	192,840	4252	2.20	175	1101.9	Total	98,809	1181	1.20	71	1391.7
AJNALA TEHSIL.						TOTAL AMRITSAR DISTRICT 1906.					
Dec. '05	—	—	—	—	—	Dec. '05	2463	68	2.76	2	1231.5
January	—	—	—	—	—	January	18,697	541	2.89	12	1558.1
February	1455	37	2.54	2	727.5	February	15,962	415	2.60	10	1596.2
March	6542	128	1.96	2	3271.0	March	64,953	1676	2.58	38	1709.3
April	2100	86	4.10	3	700.0	April	97,009	2344	2.42	97	1000.1
May	11,554	128	1.11	12	962.8	May	96,163	693	.72	92	1045.3
June	3914	3	.08	2	1957.0	June	21,967	78	.36	16	1372.9
Total	25,565	382	1.49	21	1217.4	Total	317,214	5815	1.83	267	1188.1

TABLE XLIX. *Amritsar* 1907.

AMRITSAR TEHSIL.						TARN-TARN TEHSIL.					
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '06	22,742	1222	5.37	19	1196.9	Dec. '06	5531	234	4.23	4	1382.8
January	25,491	992	3.89	19	1341.6	January	5800	181	3.12	4	1450.0
February	24,633	884	3.59	25	985.3	February	19,570	752	3.84	12	1630.8
March	78,868	2848	3.61	70	1126.7	March	44,626	1795	4.02	30	1487.5
April	59,139	2141	3.62	60	985.7	April	95,592	2232	2.33	73	1309.5
May	23,476	392	1.67	30	782.5	May	42,136	930	2.21	49	859.9
June	5976	73	1.22	8	747.0	June	5074	57	1.12	7	724.9
Total	240,325	8552	3.56	231	1040.4	Total	218,329	6181	2.83	179	1219.7
AJNALA TEHSIL.						TOTAL AMRITSAR DISTRICT 1907.					
Dec. '06	13,210	766	5.80	9	1467.8	Dec. '06	41,483	2222	5.36	32	1296.3
January	6092	403	6.62	9	676.9	January	37,383	1576	4.22	32	1168.2
February	16,520	932	5.64	13	1270.8	February	60,723	2568	4.23	50	1214.5
March	26,173	1830	6.99	25	1046.9	March	149,667	6473	4.33	125	1197.3
April	51,679	3091	5.98	62	833.5	April	206,410	7464	3.62	195	1058.5
May	37,506	1550	4.13	49	765.4	May	103,118	2872	2.79	128	805.6
June	5041	120	2.38	10	504.1	June	16,091	250	1.55	25	643.6
Total	156,221	8692	5.56	177	882.6	Total	614,875	23,425	3.81	587	1047.5

TABLE I. *Rohtak* 1905.

ROHTAK TEHSIL.						SAMPLA TEHSIL.					
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '04	24,244	815	3.36	6	4040.7	Dec. '04	15,939	889	5.56	6	2666.5
January	61,097	3904	6.39	19	3215.6	January	21,989	1668	7.59	10	2198.9
February	11,293	868	7.69	10	1129.3	February	38,694	2476	6.40	21	1842.6
March	22,766	1940	8.52	14	1626.1	March	21,022	1916	9.11	17	1236.6
April	27,646	801	2.90	22	1256.6	April	24,921	1225	4.92	19	1311.6
May	1886	36	1.91	2	943.0	May	5428	50	.92	5	1085.6
June	—	—	—	—	—	June	1095	8	.73	1	1095.0
Total	148,932	8364	5.62	73	2040.2	Total	129,148	8232	6.37	79	1634.8
JHAMAR TEHSIL.						GOHANAH TEHSIL.					
Dec. '04	12,657	599	4.73	9	1406.3	Dec. '04	10,720	1110	10.35	4	2680.0
January	6631	679	10.24	9	736.8	January	8262	1136	13.75	3	1301.3
February	4949	282	5.70	8	618.6	February	12,618	962	7.62	4	3154.5
March	17,165	889	5.18	17	1009.7	March	21,843	2335	10.69	9	2427.0
April	16,481	648	3.93	25	659.2	April	46,779	2089	4.47	30	1559.3
May	6557	81	1.24	4	1639.3	May	16,753	234	1.40	8	2094.1
June	—	—	—	—	—	June	—	—	—	—	—
Total	64,440	3178	4.93	72	895.0	Total	116,975	7866	6.72	58	2016.8
TOTAL ROHTAK DISTRICT 1905.											
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '04	63,620	3413	5.36	25	2544.8	Dec. '04	15,939	889	5.56	6	2666.5
January	97,979	7387	7.54	41	2389.7	January	21,989	1668	7.59	10	2198.9
February	67,554	4588	6.79	43	1571.0	February	38,694	2476	6.40	21	1842.6
March	82,796	7080	8.55	57	1452.6	March	21,022	1916	9.11	17	1236.6
April	115,827	4763	4.11	96	1206.5	April	24,921	1225	4.92	19	1311.6
May	30,624	401	1.31	19	1611.8	May	5428	50	.92	5	1085.6
June	1095	8	.73	1	1095.0	June	1095	8	.73	1	1095.0
Total	459,495	27,640	6.02	282	1629.4	Total	129,148	8232	6.37	79	1634.8

TABLE LI. *Rohtak* 1907.

ROHTAK TEHSIL.					SAMPLA TEHSIL.					
Month of 1st infection	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '06	526	4.56	1	5126.0	Dec. '06	6371	340	5.34	2	3185.5
January	14,724	11.61	3	4908.0	January	8548	662	7.74	4	2137.0
February	14,113	8.38	5	2822.6	February	15,170	934	6.16	7	2107.1
March	67,038	3.82	23	2914.7	March	64,098	6917	10.79	40	1602.5
April	30,417	7.47	21	1448.4	April	27,130	2344	8.64	20	1356.5
May	10,330	1.00	7	1475.7	May	12,284	205	1.67	12	1023.7
June	612	.16	1	612.0	June	1685	25	1.48	2	842.5
Total	142,360	7.260	61	2333.8	Total	135,286	11,427	8.45	87	1555.0

JHAMAR TEHSIL.					GOHANAH TEHSIL.						
Month of 1st infection	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	
Dec. '06	—	—	—	—	Dec. '06	26,632	3029	11.37	8	3329.0	
January	—	—	—	—	January	6236	769	12.33	4	1559.0	
February	839	108	12.87	1	839.0	February	26,956	2175	8.07	13	2073.5
March	2735	109	3.99	3	911.7	March	36,014	3491	9.69	19	1895.5
April	10,776	306	2.84	14	769.7	April	28,028	1823	6.50	18	1557.1
May	8500	68	.80	7	1214.3	May	11,786	267	2.27	9	1309.6
June	1063	6	.56	2	531.5	June	930	11	1.18	1	930.0
Total	23913	597	2.50	27	885.7	Total	136,582	11,565	8.47	72	1897.0

TOTAL ROHTAK DISTRICT 1907.				
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages
Dec. '06	38,129	3603	9.45	11
January	29,508	3141	10.64	11
February	57,078	4400	7.71	26
March	169,885	13,799	8.12	85
April	96,351	5220	5.42	73
May	42,900	643	1.50	35
June	4290	43	1.00	6
Total	438,141	30,849	7.04	247

TABLE LII. *Mozaffarnagar* 1904.

MOZAFFARNAGAR TEHSIL.						JANSATH TEHSIL.					
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '03	1061	6	·57	1	1061·0	Dec. '03	18,358	645	3·51	5	3671·6
January	—	—	—	—	—	January	18,315	616	3·36	7	2616·4
February	21,602	623	2·88	7	3086·0	February	13,116	445	3·39	12	1093·0
March	3453	18	·52	2	1726·5	March	42,236	1436	3·40	25	1689·4
April	15,292	289	1·89	14	1092·3	April	35,505	516	1·45	29	1224·3
May	3418	7	·20	3	1139·3	May	7336	131	1·74	6	1256·0
June	1162	1	·09	1	1162·0	June	5502	19	·35	4	1375·5
July	999	4	·40	2	499·5	July	—	—	—	—	—
Total	46,987	948	2·02	30	1566·2	Total	140,568	3808	2·71	88	1597·4
BUDHANA TEHSIL.						TOTAL MOZAFFARNAGAR DISTRICT 1904.					
Dec. '03	—	—	—	—	—	Dec. '03	19,419	651	3·35	6	3236·5
January	—	—	—	—	—	January	18,315	616	3·36	7	2616·4
February	3863	263	6·81	1	3863·0	February	38,581	1331	3·45	20	1929·0
March	12,544	113	·90	3	4181·3	March	58,233	1567	2·69	30	1941·1
April	1829	14	·77	2	914·5	April	57,720	822	1·44	46	1254·8
May	—	—	—	—	—	May	10,954	138	1·26	9	1217·1
June	—	—	—	—	—	June	6664	20	·30	5	1332·8
July	—	—	—	—	—	July	999	4	·40	2	499·5
Total	18,236	390	2·14	6	3039·3	Total	210,885	5149	2·44	125	1687·1

TABLE LIV. *Mozaffarnagar* 1906.

MOZAFFARNAGAR TEHSIL.						JANSATH TEHSIL.					
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '05	3565	140	3.93	1	3565.0	Dec. '05	9597	144	1.50	3	3199.0
January	3438	214	6.24	2	1714.0	January	8888	588	6.62	3	2962.7
February	1448	2	.14	2	724.0	February	14,826	471	3.18	7	2118.0
March	3540	88	2.49	3	1180.0	March	136	136	1.68	6	1347.5
April	5583	52	.93	4	1398.3	April	5855	46	.79	3	1951.7
May	557	1	.18	1	557.0	May	2699	1	.04	1	2699.0
June	—	—	—	—	—	June	—	—	—	—	—
Total	18,131	497	2.74	13	1394.7	Total	49,950	1386	2.77	23	2171.7
BUHANA TEHSIL.						KAIRANA TEHSIL.					
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '05	9651	177	1.83	3	3217.0	Dec. '05	2016	12	.60	2	1008.0
January	—	—	—	—	—	January	—	—	—	—	—
February	6973	424	6.08	3	2324.3	February	—	—	—	—	—
March	10,226	266	2.60	8	1278.3	March	—	—	—	—	—
April	12,154	65	.53	7	1736.3	April	1514	45	2.97	2	757.0
May	6789	36	.53	3	2263.0	May	2694	2	.07	1	2694.0
June	—	—	—	—	—	June	—	—	—	—	—
Total	45,793	968	2.11	24	1908.0	Total	6224	59	.95	5	1244.8
TOTAL MOZAFFARNAGAR DISTRICT 1906.						TOTAL MOZAFFARNAGAR DISTRICT 1906.					
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '05	24,829	473	1.91	9	2758.8	Dec. '05	24,829	473	1.91	9	2758.8
January	3428	214	6.24	2	1714.0	January	3428	214	6.24	2	1714.0
February	17,309	1014	5.86	8	2163.6	February	17,309	1014	5.86	8	2163.6
March	28,592	825	2.89	18	1588.4	March	28,592	825	2.89	18	1588.4
April	27,346	298	1.09	19	1439.3	April	27,346	298	1.09	19	1439.3
May	15,895	85	.53	8	1986.9	May	15,895	85	.53	8	1986.9
June	12699	1	.04	1	2699.0	June	12699	1	.04	1	2699.0
Total	120,098	2910	2.42	65	1817.7	Total	120,098	2910	2.42	65	1817.7

TABLE LV. *Mozaffarnagar* 1907.

MOZAFFARNAGAR TEHSIL.					JANSATH TEHSIL.				
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.
Dec. '06	24,248	920	3.79	11	2204.4	Dec. '06	55,280	2663	4.82
January	24,516	2375	9.69	11	2298.7	January	39,613	3319	8.38
February	46,588	3878	8.32	27	725.5	February	59,294	4534	7.65
March	54,417	4865	8.94	52	1046.5	March	61,561	4820	7.83
April	57,489	3396	5.91	71	809.7	April	35,311	2511	7.11
May	19,160	380	1.98	29	660.7	May	18,908	409	2.16
June	2144	16	.75	3	714.7	June	—	—	—
Total	228,562	15,830	6.93	204	1120.4	Total	269,967	18,256	6.76
BUDHANA TEHSIL.					KAIRANA TEHSIL.				
Dec. '06	1791	116	6.48	2	895.5	Dec. '06	2261	154	6.81
January	18,885	1509	7.99	5	3777.0	January	4120	295	7.16
February	24,305	1819	7.48	12	2025.4	February	3047	291	9.55
March	16,611	1751	10.54	11	1510.1	March	12,616	1590	12.60
April	32,320	1512	4.68	26	1243.1	April	35,541	2246	6.32
May	29,727	628	2.11	24	1238.6	May	12,363	535	4.33
June	6317	84	1.33	4	1579.3	June	561	22	3.92
Total	129,956	7419	5.71	84	1547.1	Total	70,509	5133	7.28

TOTAL MOZAFFARNAGAR DISTRICT 1907.

Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '06	83,580	3746	4.61	41	2038.5
January	87,134	7498	8.61	50	1742.7
February	133,234	10,522	7.90	81	1644.9
March	145,205	13,026	8.97	133	1091.8
April	160,661	9665	6.02	174	923.3
May	80,158	1952	2.44	99	809.7
June	9022	122	1.35	10	902.2
Total	698,994	46,531	6.67	588	1188.8

TABLE LVI. *Gujrat* 1904.

GUJRAT TEHSIL.						KHARJAN TEHSIL.					
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '03	—	—	—	—	—	Dec. '03	5454	176	3·23	1	5454·0
January	—	—	—	—	—	January	—	—	—	—	—
February	667	39	5·85	2	333·5	February	1665	28	1·68	2	832·5
March	16,813	710	4·22	12	1401·1	March	3509	137	3·90	5	701·8
April	27,403	812	2·96	33	830·4	April	13,441	497	3·70	13	1033·9
May	13,380	205	1·53	20	669·0	May	6182	246	3·98	10	618·2
June	769	17	2·21	3	256·3	June	5854	30	·51	7	886·3
July	—	—	—	—	—	July	620	1	·16	1	620·0
Total	59,032	1783	3·02	70	843·3	Total	36,725	1115	3·04	39	941·7
PHALIA TEHSIL.						TOTAL GUJRAT DISTRICT 1904.					
Dec. '03	8199	736	8·98	9	911·0	Dec. '03	13,653	912	6·68	10	1365·3
January	3808	1051	27·60	4	952·0	January	3808	1051	27·60	4	952·0
February	14,197	1723	12·14	10	1419·7	February	16,529	1790	10·83	14	1180·6
March	37,810	8338	22·05	51	741·4	March	58,132	9185	15·80	68	854·9
April	57,492	8410	14·63	97	592·7	April	98,336	9719	9·88	143	687·7
May	11,006	391	3·55	16	687·9	May	30,568	842	2·76	46	664·5
June	5704	97	1·70	6	950·7	June	12,327	144	1·17	16	770·4
July	—	—	—	—	—	July	620	1	·16	1	620·0
Total	138,216	20,746	15·01	193	716·1	Total	233,973	23,644	10·11	302	774·8

TABLE LVII. *Central* 1905.

GUJRAT TEHSIL.						KHARAN TEHSIL.					
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popm. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popm. of villages
Dec. '04	6923	608	9.65	10	692.3	Dec. '04	—	—	—	—	—
January	5645	432	7.65	12	470.4	January	302	3	.99	1	302.0
February	6377	537	8.42	9	708.6	February	1725	5	.29	2	862.5
March	27,821	1812	6.51	27	1030.4	March	807	265	32.84	2	403.5
April	28,671	2109	7.36	44	651.6	April	8959	372	4.15	11	814.5
May	26,875	494	1.84	34	790.4	May	7328	132	1.80	8	916.0
June	4062	21	.52	9	451.3	June	—	—	—	—	—
July	—	—	—	—	—	July	—	—	—	—	—
Total	106,374	6073	5.71	145	733.6	Total	19,121	777	4.06	24	796.7
PHALIA TEHSIL.						TOTAL GUJRAT DISTRICT 1905.					
Dec. '04	4436	153	3.45	4	1109.0	Dec. '04	11,359	821	7.23	14	811.4
January	3051	159	5.21	3	1017.0	January	8998	594	6.60	16	562.4
February	583	87	14.92	2	291.5	February	8685	629	7.24	13	668.1
March	19,852	598	3.01	23	863.1	March	48,480	2675	5.52	52	932.3
April	33,056	1216	3.68	38	869.9	April	70,686	3697	5.23	93	760.1
May	12,654	297	1.64	19	666.0	May	46,857	833	1.78	61	768.2
June	965	7	.73	2	482.5	June	5027	28	.56	11	457.0
July	—	—	—	—	—	July	—	—	—	—	—
Total	74,597	2427	3.25	91	819.7	Total	200,092	9277	4.64	260	769.6

TABLE LVIII. *Gujrat 1907.*

GUJRAT TEHSIL.						KHARAN TEHSIL.					
Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages	Month of 1st infection	Population	No. of deaths	Mortality per cent.	No. of villages	Average popn. of villages
Dec. '06	7626	61	80	2	3813.0	Dec. '06	—	—	—	—	—
January	—	—	—	—	—	January	—	—	—	—	—
February	3569	350	9.81	5	713.8	February	2333	443	18.99	2	1166.5
March	35,037	3022	8.63	45	778.6	March	23,614	3701	15.67	25	944.6
April	84,250	6208	7.48	128	658.2	April	68,216	9210	13.50	100	682.2
May	48,702	1899	3.90	101	482.2	May	51,912	2925	5.63	113	459.4
June	16,208	289	1.78	32	506.5	June	9708	255	2.63	34	285.5
July	570	21	3.68	3	190.0	July	3235	18	.56	6	539.2
Total	195,962	11,940	6.09	316	620.1	Total	159,018	16,552	10.41	280	568.5
PHALLA TEHSIL.						TOTAL GUJRAT DISTRICT 1907.					
Dec. '06	20,570	1680	8.17	14	1469.3	Dec. '06	28,196	1741	6.17	16	1762.3
January	15,824	1909	12.06	20	791.2	January	15,824	1909	12.06	20	791.2
February	34,934	5163	14.78	49	712.9	February	40,836	5956	14.59	56	729.2
March	62,139	8576	13.80	95	654.1	March	120,790	15,299	12.67	165	732.1
April	38,208	4236	11.09	66	578.9	April	190,674	19,744	10.36	294	648.6
May	238.3	160	6.71	10	238.3	May	102,997	4984	4.84	224	459.8
June	98	19	19.39	1	98.0	June	26,014	563	2.16	67	388.3
July	—	—	—	—	—	July	3805	39	1.03	9	422.8
Total	174,156	21,743	12.48	255	682.7	Total	529,136	50,235	9.49	851	621.8

TABLE LIX.

Infected villages within selected area, Gujrat 1907.

Month of 1st infection	Population	No. of deaths	No. of villages	Mortality per cent.	Average population of villages
Dec. '06	—	—	—	—	—
January	—	—	—	—	—
February	7394	1642	8	22·21	924·3
March	28,616	4992	27	17·44	1059·9
April	33,276	6503	53	19·54	627·8
May	11,692	1045	26	8·94	449·7
June	1425	89	8	6·25	178·1
Total	82,403	14,271	122	17·32	675·4

Infected villages without selected area, Gujrat 1907.

Dec. '06	28,196	1741	16	6·17	1762·3
January	15,824	1909	20	12·06	791·2
February	33,442	4314	48	12·90	696·7
March	92,174	10,307	138	11·18	667·9
April	157,398	13,241	241	8·41	653·1
May	91,305	3939	198	4·31	461·1
June	24,589	474	59	1·93	416·8
July	3805	39	9	1·02	422·8
Total	446,733	35,964	729	8·05	612·8

TABLE LX.

Infected villages within selected area, Gujrat District 1904.

Month of 1st infection	Population	No. of deaths	No. of villages	Mortality per cent.	Average population of villages
Dec. '03	5454	176	1	3·23	5454·0
January	—	—	—	—	—
February	534	18	1	3·37	534·0
March	689	209	3	30·33	229·7
April	15,146	1215	15	8·02	1009·7
May	3972	205	6	5·16	662·0
June	3383	18	4	·53	845·8
July	—	—	—	—	—
Total	29,178	1841	30	6·31	972·6

Infected villages without selected area, Gujrat District 1904.

Dec. '03	8199	736	9	8·98	911·0
January	3808	1051	4	27·60	952·0
February	15,995	1772	13	11·08	1230·4
March	57,443	8976	65	15·63	883·7
April	83,190	8504	128	10·22	649·9
May	26,596	637	40	2·40	664·9
June	8944	126	12	1·41	745·3
July	620	1	1	·16	620·0
Total	204,795	21,803	272	10·65	752·9

TABLE LXI. *Clerical mortality 1348—9.*

(1) INSTITUTIONS AND AVERAGE INSTITUTIONS.

Place	Average No. of Institutions (Annual)	Institutions 1348—9	Ratio
Cornwall & Devon ...	36	382	10·6
Hants. ...	21	228	10·9
Cambridge ...	9	97	10·8
Hereford ...	13	200	15·4
Shropshire ...	1½	29	19·3
Derby ...	7	77 (deaths)	11·0
Wilts. ...	26	176 (in 2 years)	6·8
Norwich (Diocese) ...	35	297 (in 2 years)	8·5
Bath & Wells (Diocese)	77	800	10·3

(2) TOTAL BENEFICES AND INSTITUTIONS.

			Percentage
Bucks. ...	180	83	46·1
Worcester ...	138	67	48·5
Northants. ...	281	161	57·3
Derby ...	103	77	71·3
Dorset ...	211	110	52·1
Notts. ...	126	65	51·6
Chester ...	70	30 (1 Archdeaconry)	—
W. R. Yorks. ...	141	96	68·1
E. R. Yorks. ...	126	65	51·6

TABLE LXII. *English Records.*

EPIDEMIC OF 1603—4.

City or village	Estimated population	Population required to give the same rate of mortality as London	Plague deaths
London	250,000	250,000	33,347
Chester	Less than 6000 *	12,265	1636
York	Less than 6000 †	26,329	3512
Shrewsbury	Less than 4000 ‡	5000	667

EPIDEMIC OF 1625—6.

London	320,000	320,000	41,313
Plymouth	Less than 10,000 *	15,492	2000
Ashburton	—	2827	365 §
Norwich	23,000	11,085	1431

EPIDEMIC OF 1665—6.

London	460,000	460,000	68,596
Eyam	350	1737	259
Deptford	—	3500	522
Colchester	Less than 10,000 *	32,302	4817

* "In the reign of Charles the Second no provincial town in the kingdom contained thirty thousand inhabitants and only four provincial towns as many as ten thousand inhabitants." Macaulay, I. 164.

(The four towns were Bristol, Norwich, York, Exeter.)

† Macaulay estimates the population in 1685 at about 10,000.

‡ 7000 inhabitants at the census of 1695.

§ "Probably a fourth of the inhabitants," *Notes and Queries*, 6 Ser. III. 477, cited by Creighton, I. 524.

|| 28,000—29,000 at the census of 1693.

TABLE LXIII.

The plague experience of the South of France 1720—21.

Population group	No. of towns	Total population	Total deaths	Death rate
90,000	1 (Marseilles)	90,000	39,134	43.5
20,000—90,000	2	46,000	20,694	45.0
5000—	4	24,000	5457	22.7
3000—	7	25,200	4805	19.1
2000—	3	6200	1300	21.0
1000—	4	5050	1242	24.6
500—	10	7224	1904	26.4
0—	7	2175	493	22.7

TABLE LXIV.

Deaths from plague, September 1906—September 1907.

(Grouped in 3-weekly periods.)

	Amritsar	Gujrat
—September 15th	1	—
—October 6th	4	—
—October 27th	5	18
—November 17th	35	97
—December 8th	132	59
—December 29th	346	173
—January 19th	389	201
—February 9th	639	677
—March 2nd	1109	1585
—March 23rd	1794	5766
—April 13th	3244	6417
—May 4th	5591	11,827
—May 25th	6867	15,823
—June 15th	2988	9357
—July 6th	707	2820
—July 27th	76	418
—August 17th	16	7
—September 7th	4	—
Total	23,947	55,245

Amritsar 1905—1906.

(4-weekly periods.)

—September 23rd	...	...	1
—October 21st	...	...	0
—November 18th	...	...	5
—December 16th	...	...	26
—January 13th	...	...	30
—February 10th	...	...	60
—March 10th	...	...	143
—April 7th	...	...	437
—May 5th	...	...	1896
—June 2nd	...	...	3010
—June 30th	...	...	431
—July 28th	...	...	20
—August 25th	...	...	2
Total			6061

APPENDIX IV.

Further Note on the rates of mortality in villages of different sizes.

On p. 81 I attempted to show that the difference in the rate of mortality, observed in most epidemics when one passed from large to small infected villages, could hardly be accounted for by assuming that the chance of infection reaching a colony of rats was independent and that the ratio between the number of human deaths and the number of plague-infected colonies was constant. I will here discuss the results which flow from the assumption that a single colony has a greater chance of being infected if it is situated in a large village than if it is in a small one. In other words I will assume that the chance of infection is not constant from group to group though constant within each group.

Let p_1 be the chance of a colony becoming infected among the colonies situated in villages which contain on the average m_1 colonies a piece. Similarly let p_2 and m_2 be corresponding values for another group of villages. Let $p_1 + q_1 = 1$ and $p_2 + q_2 = 1$.

Then the ratio of the mortalities among infected villages of the two groups is¹

$$\frac{1 - q_1}{1 - q_2} \times \frac{1 - q_2^{m_2}}{1 - q_1^{m_1}} \dots \dots \dots (1).$$

Since q_1 and q_2 are by definition less than unity this approaches

$$\frac{1 - q_1}{1 - q_2} \dots \dots \dots (2)$$

when m_1 and m_2 are both large. If q_1 and q_2 are fixed, (1) will be as small as possible when m_2 is unity and m_1 very large, in which case it approaches $1 - q_1 = p_1$.

The value of (1) lies accordingly between p_1 and $\frac{p_1}{p_2}$, assuming that m_2 cannot be greater than m_1 .

But we have no means of determining the values of p_1 , p_2 , m_1 and m_2 . We may presume that p_1 and p_2 are proportional to the ratio: $\frac{\text{Infected Villages}}{\text{Total Villages}}$ for each class and that m_1 is to m_2 in the ratio of the average populations of each class, but we cannot determine the absolute values¹.

Simply as an arithmetical illustration, let us suppose that in the two groups of villages having populations 1000—3000 and 0—1000, the p 's are absolutely the ratios of infected to total villages, that m_2 the

¹ Assuming that the average populations are $m_1 n$ and $m_2 n$ respectively where n is a constant.

number of colonies per village in the group 0—1000 is 1, and $m_1 = 2$ or $= 3$. Then, taking Amritsar 1907,

we have $q_1 = \frac{4.5}{2.46} = .183$ and $q_2 = \frac{4.09}{7.82} = .523$.

The ratio of mortality rate in infected villages 1000—3000 to mortality rate in infected villages 0—1000 will be .822 if $m_1 = 3$ and $m_2 = 1$, .85 if $m_1 = 2$ and $m_2 = 1$. The observed ratio is .63. The limits within which the ratio might be anticipated to fall are .817 and 1.71.

In this way, I have deduced the entries in Table LXVII from the data of Table LXV. In two cases, Amritsar 1905 and 1907, the observed ratio is smaller than the least value deduced from the above hypothesis. In the seven other illustrations the real value is between the theoretical limits, but no plausible values for p_1 , p_2 , m_1 and m_2 are suggested by the results. The general conclusion appears to be that, even as modified here, the hypothesis will not explain the facts.

TABLE LXV.

Comparison of mortality for villages actually infected with that of whole population at risk.

AMRITSAR.							
<i>Population 0—1000.</i>							
	Population of infected villages	No. of villages	No. of deaths	D.R. ‰	Population of all villages, in group	No. of villages	D.R. ‰
1902	13,151	20	298	2.27	357,961	782	.08
1903	163,074	281	10,286	6.31	357,961	782	2.87
1904	137,851	247	5177	3.76	357,961	782	1.45
1905	228,256	419	12,632	5.53	357,961	782	3.53
1906	77,707	142	2015	2.59	357,961	782	.56
1907	205,246	373	10,560	5.15	357,961	782	2.95
<i>Population 1000—3000.</i>							
1902	49,489	31	1008	2.04	378,657	246	.27
1903	275,965	175	16,163	5.86	378,657	246	4.27
1904	266,025	168	11,357	4.27	378,657	246	3.00
1905	336,362	215	13,554	4.03	378,657	246	3.58
1906	167,467	109	2785	1.66	378,657	246	.74
1907	318,149	201	10,370	3.26	378,657	246	2.74
ROHTAK.							
<i>Population 0—1000.</i>							
1904	12,604	23	376	2.98	137,080	299	.27
1905	65,434	120	4281	6.54	137,080	299	3.12
1906	911	2	9	.99	137,080	299	.01
1907	54,446	91	3224	5.92	137,080	299	2.35
<i>Population 1000—3000.</i>							
1904	27,432	17	619	2.26	251,662	152	.25
1905	197,942	119	11,751	5.94	251,662	152	4.67
1906	23,200	13	748	3.22	251,662	152	.80
1907	192,660	112	13,487	7.00	251,662	152	5.36

TABLE LXVI.

Comparison of mortality for villages actually infected with that of whole population at risk.

MOZAFFARNAGAR.

Population 0—3000.

	Population of infected villages	No. of villages	No. of deaths	D.R. ‰	Population of all villages, in group	No. of villages	D.R. ‰
1902	2137	1	3	·14	645,492	924	·00
1903	23,435	18	525	2·24	645,492	924	·08
1904	126,228	108	3115	2·47	645,492	924	·48
1905	298,663	273	9285	3·11	645,492	924	1·44
1906	70,485	54	1551	2·20	645,492	924	·24
1907	489,201	541	33,544	6·86	645,492	924	5·20

Population over 3000.

1902	23,444	1	1	·00	274,710	52	·00
1903	50,509	5	257	·51	274,710	52	·09
1904	131,227	20	2371	1·81	274,710	52	·86
1905	247,026	43	6961	2·82	274,710	52	2·53
1906	103,924	14	1526	1·47	274,710	52	·56
1907	264,202	49	16,365	6·19	274,710	52	5·96

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Population 0—3000.

1898	11,635	15	352	3·03	952,181	2085	·04
1899	3701	4	9	·24	952,181	2085	·00
1900	9636	11	50	·52	952,181	2085	·01
1901	33,760	43	332	·98	952,181	2085	·03
1902	340,675	464	10,824	3·18	952,181	2085	1·14
1903	No data :	—	—	—	952,181	2085	—
1904	506,168	844	24,642	4·87	952,181	2085	2·59
1905	555,936	940	18,079	3·25	952,181	2085	1·90
1906	176,690	284	3513	1·99	952,181	2085	·37
1907	530,305	879	14,745	2·78	952,181	2085	1·55

Population over 3000.

1898	5803	1	87	1·50	83,633	16	·10
1899	—	—	—	—	83,633	16	—
1900	—	—	—	—	83,633	16	—
1901	9030	2	112	1·24	83,633	16	·13
1902	63,755	12	864	1·36	83,633	16	1·03
1903	No data :	—	—	—	83,633	16	—
1904	65,952	12	1425	2·16	83,633	16	1·70
1905	63,305	12	1052	1·66	83,633	16	1·26
1906	52,038	9	612	1·18	83,633	16	·73
1907	68,079	13	962	1·41	83,633	16	1·15

TABLE LXVII.

*Ratio of the rate of mortality in infected villages 1000—3000,
to the rate in infected villages 0—1000.*

District	Year	(1)	(2)	(3)	(4)	(5)
		Observed ratio	Smallest theoretical	Largest theoretical	Theoretical for $m_1=3, m_2=1$	Theoretical for $m_1=2, m_2=1$
Amritsar	1902	·90	·13	4·93	·38	·53
„	1903	·93	·71	1·98	·73	·78
„	1904	1·13	·68	2·16	·71	·76
„	1905	·73	·87	1·63	·88	·89
„	1906	·64	·44	2·44	·54	·64
„	1907	·63	·82	1·71	·82	·85
Rohtak	1904	·76	·11	1·45	·37	·53
„	1905	·91	·78	1·95	·79	·82
„	1907	1·18	·74	2·42	·75	·79

AN EXAMINATION OF SOME FACTORS INFLUENCING THE RATE OF INFANT MORTALITY.

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I. Introduction.

IN all countries which make statistical returns, attention has been directed to the large proportion of children failing to complete one year of life. Even the most highly civilised races lose in this way more than ten per cent. of their potential citizens as will appear from the data contained in the following table (*Deutsche Sterbetafeln*, 1910).

Country				Number out of 100,000 born who survive the first year
Sweden	...	...	...	88,917
France	...	...	...	83,674
Belgium	...	...	...	83,114
England and Wales	...	...	...	82,814
Massachusetts	...	...	...	82,767
Holland	...	...	...	82,681
Italy	...	...	...	82,481
German Empire	...	...	...	76,614
Austria	...	...	...	75,028

The significance of this phenomenon, its advantage or disadvantage to the state, has been variously interpreted by writers on the subject. Some have argued that a high rate of infant mortality may be of advantage, that persons ill-adapted to their social and physical environment are in this way eliminated with a minimum of individual suffering and material inconvenience to the community. Others, we think the

majority, have taught that a high infant death rate is detrimental, that the elimination even if selective does not necessarily remove those calculated to prove inferior citizens.

Whether the infant death rate is selective and, if selective, whether such a process is really beneficial to the state are questions we do not propose to discuss at length, although some slight reference to the subject will be unavoidable in connection with the concluding passages in our memoir; we shall be mainly occupied with certain preliminary topics. If the factors determining the rate of infant mortality are within our control, the consequences of exercising that control may well be considered; if, on the other hand, the factors are beyond the scope of human interference, such a discussion would be of purely speculative interest. At one time, it was thought that a high infant death rate was part of the established order of nature, as it were, inherent in the necessary constitution of society. In the famous essay of Malthus (1817) this view finds adequate expression. Thus, he writes (Vol. II. p. 49) "And yet when we contemplate the insufficiency of the price of labour to maintain a large family, and the amount of mortality which arises directly and indirectly from poverty; and add to this the crowds of children, which are cut off prematurely in our great towns, our manufactories and our workhouses; we shall be compelled to acknowledge that if the number born annually were not greatly thinned by this premature mortality, the funds for the maintenance of labour must increase with much greater rapidity than they have ever done hitherto in this country in order to find work and food for the additional numbers that would then grow up to manhood."

According to Malthus fifty per cent. of all persons born died in London under the age of three years, in Vienna and Stockholm under two, in Manchester and Norwich under five and in Northampton under ten (Vol. II. 58). In the whole of Sweden the average mortality in the first year was 25 per cent. (I. 421). (For a discussion of older natality and mortality statistics, see Wernicke, 1889.)

At the present time, heavy as is the toll taken of infant lives, it is markedly less than indicated in the figures just mentioned and most writers have attributed the reduction to improved environmental conditions, using that word in a broad sense. We must, however, remember that not only has sanitary science advanced but, in addition, the birth rate has fallen. Were Malthus alive now, he would probably attribute a large share in the diminution of the Infant Death Rate to the fall in the Birth Rate and it is important to learn how far such an

attribution would be correct, since the Birth Rate is hardly amenable to direct administrative control.

Numerous collections of data have been published with the object of determining which of the various factors may be responsible for the observed result; it is to this question which we desire to devote ourselves.

Since what may be called the environmental factor is capable of resolution into many components, it will be seen that the problem is too complex for any satisfactory solution to be reached without a minute and somewhat difficult analysis. It has seemed to us probable that an application of modern statistical methods to existing collections of data might lead to results suggesting further lines of inquiry, and the present essay is the result of such an attempt. Evidently no single group of workers can possibly deal with a tithe of the accumulated facts, but any contribution, however slight, may be of service to those called upon to face one of the most serious problems falling within the scope of Preventive Medicine.

II. *Nature of the Material Studied.*

Our work is based upon a statistical reduction of certain figures contained in an important memoir by Dr Alfred Groth and Prof. Martin Hahn, entitled *Die Säuglingsverhältnisse in Bayern*¹. We shall therefore first describe the nature of these authors' inquiry, the material they collected and the conclusions they were led to adopt. The authors set themselves the task of elucidating the connections between Infant Mortality, the Methods of Infant feeding, the Birth Rate, the Degree of Poverty and the Fitness of Recruits for Military Service in different parts of the kingdom of Bavaria. In their first section they publish tables giving the population, the birth rate and the death rate of infants for the provinces of Bavaria from 1835-1904. From these figures various conclusions are drawn which do not concern us for the moment. They then pass on to the state of affairs in Bavaria between 1900-4. This part of the memoir deals with the inter-relations of Infant Mortality, Artificial Feeding of Infants, the Birth Rate and the Poverty Rate. For the purposes of examination the data are grouped under Regierungsbezirke (Oberbayern, Niederbayern, Pfalz, Oberpfalz, Oberfranken, Mittelfranken, Unterfranken, Schwaben) and a further

¹ Cited in future as Groth and Hahn.

subdivision is effected into Unmittelbaren Städte and Bezirksamter¹. With regard to this latter distinction, the authors write (p. 14) "Die unmittelbaren Städte, die zwar von verschiedener Grösse doch nur ausnahmsweise weniger als 5,000, in den meisten Fällen wesentlich mehr Einwohner besitzen, treten einmal durch das ihnen eigene engere Zusammenwohnen auf kleinerem Terrain und dann durch die Zusammensetzung ihrer Bewölkerung aus verschiedenen sozialen Schichten in Gegensatz zu den das Land in engeren Sinne darstellenden Bezirksamtern. Wenn auch die letzteren sehr häufig Städte mit einigen tausend Bewohnern in sich beschliessen, so lassen dieselben doch mehr den Charakter des sie umgebenden flachen Landes durch ihre mehr oder weniger weitgehende Abhängigkeit von demselben erkennen."

The exact degree of differentiation here indicated cannot be profitably discussed by foreign critics. Thus, it is not clear how the delimitation is effected, *e.g.* we have Unmittelbare Stadt München with a population of 519,162 and the Bezirksamt München with 44,092. This hardly suggests that the Bezirksamt corresponds to a suburban zone unless the tendency to the decentralisation of dwellings on the part of urban populations, which is so marked in England, does not exist in Bavaria. On the whole, however, it may reasonably be presumed that a Bezirksamt corresponds to an English Rural District and an Unmittelbare Stadt to what we term an Urban District.

We now come to the variables investigated.

(1) *Population*. "Die mittleren Bevölkerungszahlen der einzelnen Verwaltungsbezirke wurden dadurch gewonnen, dass einmal die für das Volkszählungsjahr 1900 erhobenen und in dem Gemeindeverzeichnisse des Königreichs Bayern niedergelegten Zahlen und dann die des darauffolgenden Zählungsjahres, nämlich des Kalenderjahres 1905 zur ihrer Berechnung herangezogen wurden" (p. 14).

(2) *Birth Rate*. This is the number of live-born children per cent. of the whole population (1900-1904).

(3) *Infant Death Rate*. The number of deaths in the first year of life per hundred live-born children (1900-1904).

(4) *Poverty*. Consultation with the officials of the Finance Ministry having convinced the authors that no satisfactory measure could be derived from taxation statistics, they decided to use the percentage of persons in receipt of public assistance (aus öffentlichen Mitteln unterstützte Personen).

¹ In the Pfalz, the sixteen administrative districts are not separated into towns and country districts neither are poverty statistics (*vid. infra*) given.

Groth and Hahn frankly admit that this method of estimating the economic state of a district is open to serious objections. Thus, it might well be that the extent of public assistance provided depends to some extent on the amount of money which can be raised locally, so that a rich district may give more assistance than a poor one although the latter has proportionally a greater number of necessitous inhabitants. In mitigation, they urge that this factor will rather influence the *amount* expended than the *number of persons* relieved (Groth and Hahn, p. 25). Another objection is that the poverty statistics are based on the years 1899–1902, not on the period (1900–4) to which the birth and death rates refer. That the criterion of poverty is insufficient will be recognised, but, as will be shown later on, constants based upon it bear comparison with results obtained in a better way.

(5) *The method of feeding.* Records of the extent to which the use of artificial food prevailed were obtained in a simple way. Inquiries were directed to the officers in charge of the public vaccination departments and a census made of the form of nourishment provided for the infants brought up to be vaccinated. In this way information was obtained respecting a large sample of infants in each district. Thus the smallest total upon which a percentage figure is based (in Bezirksämter) was 160, the largest 2048, and the average 628.

No doubt this method is not calculated to yield complete information regarding the prevalence of artificial feeding since, *inter alia*, it may be presumed that a considerable proportion of the wealthier inhabitants do not bring their children to the public vaccinator at all. At the same time, the value of such extensive sampling from the standpoint of the statistician and publicist is considerable. Unfortunately, as in the case of the poverty statistics, the years of record do not correspond to those used for the birth-rate, nor are they the same in all districts (1905–8). A more formidable objection, deduced from statistical considerations, will be mentioned below.

The methods adopted by Groth and Hahn in reducing their material would not altogether commend themselves to an English statistician. Much use is made of summarising tables, of which the following is an example (Groth and Hahn, p. 27).

Diagrams have also been constructed to show the relations between the variables, but are not of a nature permitting accurate comparison.

In the concluding section of their paper, Groth and Hahn compare (1) Percentage mortality in the 1st year of life; (2) Percentage of Infants not breast fed; (3) Percentage of recruits examined and declared

*Poverty and Infant Mortality, mean value per 29 Bezirksämter
arranged according to the number of paupers, 1899-1902.*

	Teuschnitz to Aschaffenburg	Mellrichstadt to Münchberg	Eggenfelden to Günzburg	Hersbruck to Hilpoltstein	Dillingen to Graffenau
Mean Poverty- Figure	1.38	1.88	2.32	2.79	3.65
Mean Infant Mor- tality-Figure	20.9	20.8	26.4	27.4	30.4

fit; (4) Mortality at ages 6-10; (5) Mortality at ages 11-20. Comparisons are effected as in the earlier chapters of the work.

We shall now briefly summarise the main conclusions which Groth and Hahn adopt on the basis of their enquiry.

The height of the infant mortality rate depends on the combined working of many factors, the separate influence of which ought neither to be under- nor over-emphasized. In particular, it is inaccurate to delimit a small group of so-called primary factors and to contrast them with secondary influences. "The complete examination of the infantile mortality of a race cannot be separated from the coincident study of the height of the birth rate and the number of children which survive the second year through the inter-play of birth and death" (Groth and Hahn, p. 81).

"The power of procreation does not determine the height of the birth-rate but, in the first place, the effective will of the people, increasing with improved material conditions and social insight, to limit the number of children (artificial limitation)" (*op. cit.* p. 81). The authors regard prolonged lactation as an anti-conceptual influence of power commensurate with the "heutzutage weitverbreitete Herabsetzung der Kinderzahl auf unnatürlichem Wege." They consider that the fall in the birth rate of recent years in Bavaria is to be attributed to the improvement of hygienic and social conditions which have accompanied it.

The writers further hold that breast feeding can almost neutralise (so gut wie vollkommen zu paralysieren) the influence of an unfavourable economic environment on the rate of infant mortality. They emphasize the necessity of caution in estimating the nature of the connection between infant mortality and its supposed factors, remarking that the relation between the infant birth and death rates is "too complex and too indirect to allow of its being set out in a general stereotyped way." Groth and Hahn would appear to regard the

method of feeding as the most influential *single* factor at work, in particular as of more importance than general hygienic measures. They also find a low infant death rate and a high percentage of breast fed children associated with a high degree of military fitness, although the correspondence was not invariable.

It must, we think, be recognised that Drs Groth and Hahn have made an important contribution to the literature of their subject. It is, indeed, because their material, whatever may be said of its shortcomings, seems peculiarly far-reaching that we have made it the foundation of our analysis. Where, in our judgment, the paper needs extension is in the analysis of details; since what the hygienist requires is exact information regarding the relative importance of the factors displayed. Neither by summarising tables nor by diagrams is it possible to extract from the figures all the information which they should be compelled to furnish; a more refined statistical calculus is imperatively required¹.

We do not suggest that the method about to be described is perfect or that the conclusions we have reached supersede those of Groth and Hahn. We believe, however, it will be found that we have been able to place some important results in a clearer light than was possible with the simpler methods employed by the original authors.

III. *Analysis of the Bavarian data.*

We now pass to a more detailed analysis of Groth and Hahn's material. Confining ourselves in the first instance to the subject of infant mortality without referring to its effect upon adult fitness, which cannot indeed be deduced from the figures, we have four variables to consider: (1) The Infant Mortality Rate, (2) The Birth Rate, (3) The Poverty Rate, (4) The Artificial Feeding Rate. We have already indicated some criticisms to which the actual data are subject. It is at once clear that a distinction must be drawn between rural and urban districts, that the data must be divided into two classes. Our first proceeding was to calculate the correlations between the variables, taken in pairs, for the two classes separately.

Table I contains the preliminary results obtained².

¹ On a subsequent page we call attention to uncertainties regarding the material suggested by our examination.

² In working, more decimal places were retained (not less than five) than might perhaps be inferred from the tabled results.

Rate of Infant Mortality

TABLE I.

Crude Correlations (Bavaria).

TOWNS.			
Variables		No. of observations	Coefficient of correlation
Birth Rate and Artificial Feeding Rate	...	20	$-.258 \pm .141$
Poverty and Artificial Feeding Rates	...	20	$.324 \pm .135$
Birth and Poverty Rates		42	$.045 \pm .104$
Birth and Infant Mortality Rates		42	$-.004 \pm .104$
Poverty and Infant Mortality Rates	...	42	$.122 \pm .103$
Artificial Feeding and Infant Mortality Rates		20	$.770 \pm .062$
RURAL DISTRICTS.			
Birth and Artificial Feeding Rates	...	97	$.397 \pm .058$
Poverty and Artificial Feeding Rates	...	87	$.369 \pm .062$
Birth and Poverty Rates		145	$.423 \pm .046$
Birth and Infant Mortality Rates		161	$.719 \pm .018$
Poverty and Infant Mortality Rates	...	145	$.465 \pm .042$
Artificial Feeding and Infant Mortality Rates		97	$.761 \pm .029$

The probable errors of the coefficients being, in the case of the urban districts, very large, little importance can be attached to the results: it would appear, however, to judge for example from the Birth Rate-Artificial Feeding coefficients, that the groups are not comparable. In what follows, attention will be exclusively directed to the Bezirksämter. (There are numerous papers especially devoted to urban Infant Mortality, *e.g.* Silbergleit, 1897.)

TABLE II.

Variables			Coefficient of correlation
Population and B. R.	...	...	$.146 \pm .053$
Population and Pov. R.	...	...	$.091 \pm .056$
Population and Inf. M. R.	...	...	$-.068 \pm .053$
Population and Art. F. R.	...	...	$-.080 \pm .068$

TABLE III.

Total Coefficients for the Rural Districts corrected for Population.

Variables			Coefficient of correlation
B. R. and Art. F. R.	...	...	$.414 \pm .057$
Pov. R. and Art. F. R.	...	...	$.379 \pm .062$
B. R. and Pov. R.	...	...	$.416 \pm .046$
B. R. and Inf. M. R.	...	...	$.738 \pm .017$
Pov. R. and Inf. M. R.	...	...	$.475 \pm .042$
Art. F. R. and Inf. M. R.	...	...	$.760 \pm .029$

To begin with, it has probably occurred to the reader that the question of spurious correlation arises, owing to the fact (*inter alia*) that the absolute population differs extremely in the various districts. It seemed, therefore, desirable to eliminate so far as possible this cause of error by calculating the correlation between absolute size and each variable, then determining the (partial) correlation with the population factor constant. Table II contains the values of the correlation between absolute size and each rate. Table III shows the result for each coefficient of keeping the population constant.

The next step was to determine the *net* or partial correlation between the variables taken in pairs for a constant value of the other two characters. In working out these coefficients, the notation introduced by Yule (Yule, 1907 and 1911) is very satisfactory and permits the arithmetical work to be so arranged that a rapid check upon its accuracy is secured. We have accordingly in every case involving four variables used this method. The results so far attained appear in Table IV.

TABLE IV.

Partial Correlations: Bavarian Rural Districts (Population Constant).

Variables	Constants	Correlation
Birth Rate and Art. Feeding Rate	Poverty Rate and Infant Mort. Rate	$-.341 \pm .064$
Poverty Rate and Art. Feeding Rate	Birth Rate and Infant Mort. Rate	$.074 \pm .072$
Birth Rate and Poverty Rate ...	Art. Feeding Rate and Infant Mort. Rate	$.128 \pm .071$
Birth Rate and Infant Mort. Rate	Poverty Rate and Art. Feeding Rate	$.687 \pm .038$
Poverty Rate and Infant Mort. Rate	Birth Rate and Art. Feeding Rate	$.136 \pm .071$
Art. Feeding Rate and Infant Mort. Rate	Birth Rate and Poverty Rate ...	$.721 \pm .035$

The next step in our investigation was to determine whether the coefficients could be substantially altered by excluding the returns from the Bavarian Palatinate (Pfalz) since in the case of this province there is no separation into rural and urban districts. The whole of the previous calculations were accordingly repeated on the data reduced in this way (Table V). It will be noticed that some of the absolute values have been changed but not to an extent seriously affecting the interpretation of Table IV.

We next faced the difficulty introduced by the fact that Groth and Hahn calculated the birth rate upon the total population, not upon the numbers of women at fertile ages in the districts. Since it might be

Rate of Infant Mortality

that marked local differences in age or sex constitution existed, this proceeding was open to criticism. It was not possible, with the aid of any published material, to remedy the defect, but—by the courtesy of Dr Zahn, Director of the Royal Bavarian Statistical Office, to whom we desire to tender our hearty thanks—we have been supplied with the numbers of women aged 16–50 enumerated in each district for the

TABLE V.

Correlation between Infant Mortality Rate, Poverty Rate, Birth Rate and Artificial Feeding Rate in Bavarian Rural Districts excluding Bavarian Palatinate (Pfalz).

Variables	No. of obs.	Correlation
Birth Rate and Art. Feeding Rate ...	87	$\cdot 428 \pm \cdot 059$
Birth Rate and Poverty Rate ...	145	$\cdot 423 \pm \cdot 046$
Poverty Rate and Art. Feeding Rate	87	$\cdot 369 \pm \cdot 063$
Birth Rate and Infant Mort. Rate ...	145	$\cdot 790 \pm \cdot 014$
Poverty Rate and Infant Mort. Rate	145	$\cdot 465 \pm \cdot 042$
Art. Feeding Rate & Infant Mort. Rate	87	$\cdot 727 \pm \cdot 034$

Correlation of Population with each of the Variables.

Birth Rate and Population ...	...	...	$\cdot 157 \pm \cdot 056$
Poverty Rate and Population	...	...	$\cdot 091 \pm \cdot 056$
Infant Mortality Rate and Population	...	...	$\cdot 250 \pm \cdot 052$
Artificial Feeding Rate and Population	...	...	$\cdot 204 \pm \cdot 069$

Total Correlations with Population Constant.

Birth Rate and Art. Feeding Rate ...	...	...	$\cdot 410 \pm \cdot 059$
Birth Rate and Poverty Rate	...	...	$\cdot 415 \pm \cdot 046$
Poverty Rate and Art. Feeding Rate	...	...	$\cdot 359 \pm \cdot 063$
Birth Rate and Infant Mortality Rate	...	...	$\cdot 785 \pm \cdot 012$
Poverty Rate and Infant Mortality Rate	...	...	$\cdot 459 \pm \cdot 042$
Art. Feeding Rate and Infant Mortality Rate	...	...	$\cdot 713 \pm \cdot 036$

Partial Correlations with Population Constant.

Variables	Constants	Correlation
Birth Rate and Art. Feeding Rate	Poverty Rate and Infant Mort. Rate	$-\cdot 354 \pm \cdot 063$
Birth Rate and Poverty Rate ...	Art. Feeding Rate and Infant Mort. Rate	$\cdot 126 \pm \cdot 071$
Poverty Rate and Art. Feeding Rate	Birth Rate and Infant Mort. Rate	$\cdot 092 \pm \cdot 072$
Birth Rate and Infant Mort. Rate	Poverty Rate and Art. Feeding Rate	$\cdot 746 \pm \cdot 032$
Poverty Rate and Infant Mort. Rate	Birth Rate and Art. Feeding Rate	$\cdot 111 \pm \cdot 071$
Art. Feeding Rate and Infant Mort. Rate	Birth Rate and Poverty Rate ...	$\cdot 676 \pm \cdot 039$

census year 1900¹. Since the births were averaged from 1900 to 1904, a rate calculated on the number of women recorded in 1900 would not be accurate. However, unless rapid and heterogeneous changes have taken place in the districts, such a rate should be sufficient for the present object. In Tables VI and VII the results of re-calculation upon this basis are recorded. On examination of the partial correlations it will be seen that the *relative* values are little affected.

TABLE VI.

Correlation of Infant Mortality, Artificial Feeding, Poverty and Birth Rates in Rural Districts of Bavaria including Bavarian Palatinate (Pfalz).

Birth Rate based on number of women aged 16-50.

Variables	No. of observations	Correlation
Birth Rate and Artificial Feeding Rate ...	92	$\cdot462 \pm \cdot055$
Birth Rate and Poverty Rate ...	142	$\cdot426 \pm \cdot046$
Birth Rate and Infant Mortality Rate ...	156	$\cdot696 \pm \cdot028$
Poverty Rate and Artificial Feeding Rate ...	87	$\cdot369 \pm \cdot062$
Poverty Rate and Infant Mortality Rate ...	145	$\cdot465 \pm \cdot044$
Artificial Feeding Rate and Infant Mortality Rate	97	$\cdot761 \pm \cdot029$

Correlation between Population and each of the Variables.

Birth Rate and Population ...	156	$\cdot105 \pm \cdot053$
Poverty Rate and Population ...	145	$\cdot091 \pm \cdot056$
Infant Mortality Rate and Population ...	161	$-\cdot068 \pm \cdot053$
Artificial Feeding Rate and Population ...	97	$-\cdot083 \pm \cdot068$

Total Correlations: Population Constant.

Birth Rate and Artificial Feeding Rate ...	92	$\cdot474 \pm \cdot055$
Birth Rate and Poverty Rate ...	142	$\cdot420 \pm \cdot047$
Birth Rate and Infant Mortality Rate ...	156	$\cdot709 \pm \cdot027$
Poverty Rate and Artificial Feeding Rate ...	87	$\cdot379 \pm \cdot062$
Poverty Rate and Infant Mortality Rate ...	145	$\cdot475 \pm \cdot043$
Artificial Feeding Rate and Infant Mortality Rate	97	$\cdot760 \pm \cdot029$

For the purposes of Tables V-VII, the partial coefficients have been based upon the total correlations deduced from all the available data, the probable errors being calculated on the assumption that $n = 87$ (87 is the smallest number of pairs upon which any total correlation was based). If the fact that while 156 pairs are available for calculating

¹ It would probably have been better to ask for the numbers of *married* women between the ages 16-50, and we regret not to have done so (the illegitimate birth rate although high might perhaps have been neglected); but, for reasons which will appear, the correction is of less importance than would have been the case had we been working with English Registration Counties as units of grouping.

TABLE VII.

Partial Correlations: Population Constant.

Variables	Constants	Correlation
Birth Rate and Art. Feeding Rate	Poverty Rate and Infant Mort. Rate	$-.145 \pm .071$
Birth Rate and Poverty Rate ...	Infant Mort. Rate and Art. Feeding Rate	$.141 \pm .071$
Birth Rate and Infant Mort. Rate	Poverty Rate and Art. Feeding Rate	$.569 \pm .049$
Poverty Rate and Art. Feeding Rate	Birth Rate and Infant Mort. Rate	$.052 \pm .072$
Poverty Rate and Infant Mort. Rate	Birth Rate and Art. Feeding Rate	$.172 \pm .070$
Art. Feeding and Infant Mort. Rate	Birth Rate and Poverty Rate ...	$.662 \pm .041$

the Birth-Rate—Infant Death Rate correlation—only 87 give the Poverty and Artificial Feeding Rates were due to chance, this might be regarded as a sound method. The ordinary process of deducing the probable error (see Pearson and Filon, Yule 1907 and Heron 1910) is not, however, directly applicable and the procedure adopted seems open to criticism. We have accordingly recalculated the whole of the coefficients using only the districts, 84 in number, for which each of the four variables is recorded. In Table VII A, the results of this work are given from which it will be seen that, while the correlations between the Infant Mortality Rate and the Rates of Artificial Feeding and Birth are not very seriously altered, the relative importance of the Poverty Rate, as indicated by its partial correlation with the Infant Death Rate, is sensibly different. This result, together with the evidence presented further on, makes it probable that some at least of the data are not random samples in the statistical sense and emphasises the necessity of caution in drawing conclusions.

We have now detailed the leading results of our inquiry but, before entering upon a consideration of them, several points need attention. As we stated in the introduction, uncertainty exists with respect to the value of the standards of measurement employed. While we have already noticed some objections and the answers to them, further evidence remains for consideration. This consists in a comparison of the results yielded by applying similar methods of analysis to other data and is probably the most valuable kind of check which can be used. Since few, if any, observers other than Groth and Hahn have tabled information respecting all four variables in a manner suitable to our method of study, the comparison can hardly be extended beyond the total coefficients; these however can be brought into relation with several other results which will now be noticed systematically.

TABLE VII A.

Correlations between Birth Rate ($\%$ Females 16-50), Infant Mortality Rate, Poverty Rate and Artificial Feeding Rate in the 84 Rural Districts of Bavaria in which each Variable is recorded.

Variables	Correlation
Birth Rate ($\%$ Females 16-50) and Artificial Feeding Rate	$\cdot 461 \pm \cdot 058$
Birth Rate ($\%$ Females 16-50) and Poverty Rate	$\cdot 413 \pm \cdot 061$
Birth Rate ($\%$ Females 16-50) and Infant Mortality Rate	$\cdot 780 \pm \cdot 029$
Poverty Rate and Artificial Feeding Rate	$\cdot 356 \pm \cdot 064$
Poverty Rate and Infant Mortality Rate	$\cdot 526 \pm \cdot 053$
Artificial Feeding Rate and Infant Mortality Rate	$\cdot 727 \pm \cdot 035$
Birth Rate ($\%$ Females 16-50) and Population	$\cdot 154 \pm \cdot 072$
Poverty Rate and Population	$\cdot 020 \pm \cdot 074$
Infant Mortality Rate and Population	$\cdot 265 \pm \cdot 068$
Artificial Feeding Rate and Population	$\cdot 251 \pm \cdot 069$

Total Correlations with Constant Population.

Birth Rate ($\%$ Females 16-50) and Artificial Feeding Rate	$\cdot 442 \pm \cdot 059$
Birth Rate ($\%$ Females 16-50) and Poverty Rate	$\cdot 414 \pm \cdot 061$
Birth Rate ($\%$ Females 16-50) and Infant Mortality Rate	$\cdot 776 \pm \cdot 029$
Poverty Rate and Artificial Feeding Rate	$\cdot 363 \pm \cdot 064$
Poverty Rate and Infant Mortality Rate	$\cdot 549 \pm \cdot 052$
Artificial Feeding Rate and Infant Mortality Rate	$\cdot 708 \pm \cdot 037$

Partial Correlations with Constant Population.

Variables	Constants	Correlations
Birth Rate ($\%$ Females 16-50) and Art. Feeding Rate	Poverty Rate and Infant Mort. Rate	$-\cdot 240 \pm \cdot 069$
Birth Rate ($\%$ Females 16-50) and Poverty Rate	Infant Mort. Rate and Artificial Feeding Rate	$-\cdot 016 \pm \cdot 074$
Birth Rate ($\%$ Females 16-50) and Infant Mort. Rate	Poverty Rate and Artificial Feeding Rate	$\cdot 697 \pm \cdot 038$
Poverty Rate and Art. Feed'g Rate	Birth Rate and Infant Mort. Rate	$-\cdot 035 \pm \cdot 074$
Poverty Rate and Infant Mort. Rate	Birth Rate and Art. Feeding Rate	$\cdot 320 \pm \cdot 066$
Art. Feeding Rate and Infant Mort. Rate	Birth Rate and Poverty Rate	$\cdot 622 \pm \cdot 045$

The Correlation between the Birth Rate and the Infant Death Rate.

That a high birth rate tends to be associated with a high rate of infant mortality has long been recognised and we do not attempt to name all the writers who have commented upon the fact. In addition to the remarks of Malthus already quoted (see also Malthus, I. 469) we

may refer to Wappäus (1859, I. 216; II. 321), Geissler (1885), Ganzen-Müller (1897), Mombert (1907), and Prinzing (1907). We shall restrict ourselves to a few of the papers containing material which can be analysed by the methods we have employed. The principal difficulty encountered in arriving at comparable results was the non-uniformity of the standard adopted in calculating birth rates. Most writers have contented themselves with expressing births in percentages of the whole population (crude birth rate); others base their ratio upon the number of wives at fertile ages or the number of women, married and single, at those ages. Newsholme and Stevenson (1906) have pointed out the errors which may result from a comparison of crude birth rates and have introduced a valuable process of correction similar to that universally adopted in the case of deaths. We are not indeed concerned with the birth rate as a measure of absolute fertility, but it is material to know whether the index employed has the same significance from district to district; if not, the coefficient of correlation will be misleading. If we determine the correlation between the birth rate and the infant mortality rate for English Registration Counties, the method of reckoning the former makes an enormous difference in the result¹. Thus, taking the figures of Newsholme and Stevenson's paper, the correlation was .81 when the crude birth rate was used, .64 when the birth rate was expressed in terms of women between 15 and 45, .53 if the rate was in terms of married women between 15-45 and only .36 if Newsholme and Stevenson's standard birth rate was adopted. Now in the case of the Bavarian Rural Districts, the reduction of the coefficient effected when we passed from the crude birth rate to the rate in terms of all women between 16-50 was much less than for the English Registration Counties. It seemed to us that the explanation might be found in a more uniform distribution of sexes and ages in the rural districts of Bavaria than in the large and somewhat heterogeneous Registration Counties of England. As a matter of fact, we found that the coefficient of variation for the proportion of women (15-45) in the Registration Counties was 6.21; the same constant for the women (16-50) in the Bavarian Rural Districts was 3.79. The difference, $2.42 \pm .47$, is statistically significant and goes far to explain the result; since, were the age, sex, and civil state proportionally the same in all districts, the correlation would be unaffected by the method of calculating the birth rate. For these reasons, while admitting the inferiority of our standard to that of Newsholme and Stevenson, we are inclined to doubt whether the correlation is in

¹ We have not corrected for absolute population except in the case of Bavaria.

reality far from the true value. We are disposed to believe that Newsholme's result (Newsholme, 1911 ; Local Government Board, 1910, p. 49), although large and significant may be too small, an English Registration County being, from many points of view, an unsatisfactory unit—for instance, it means the grouping together of towns and country districts, which we have shown to be undesirable¹.

TABLE VIII.

Correlation between Birth Rate and Infant Death Rate.

	Source of Data	Coefficient of correlation	Computer	Remarks
27	Metropolitan Boroughs (Heron)	$\cdot 50 \pm \cdot 10$	Heron	Birth Rate based on married women (15-54).
17	Countries (Powys) ...	$\cdot 93 \pm \cdot 02$	Powys	Crude Birth Rate.
38	Cities and Towns of Massachusetts (State Board of Health) 1896	$\cdot 42 \pm \cdot 09$	Greenwood & Brown	" " "
704	Rural Districts of the German Empire (Würzburg)	$\cdot 58 \pm \cdot 02$	" "	" " "
161	Bavarian Rural Districts (Groth and Hahn)	$\cdot 72 \pm \cdot 02$	" "	" " "
156	" " "	$\cdot 68 \pm \cdot 03$	" "	Birth Rate based on all women (16-50).
44	English Registration Counties (Newsholme and Stevenson) 1903	$\cdot 36 \pm \cdot 09$	Newsholme (p. 49)	Corrected Birth Rate (Newsholme and Stevenson).
44	" " "	$\cdot 53 \pm \cdot 07$	Greenwood & Brown	Birth Rate based on wives (15-45)
44	" " "	$\cdot 64 \pm \cdot 06$	" "	Birth Rate based on all women (15-45).
44	" " "	$\cdot 81 \pm \cdot 04$	" "	Crude Birth Rate.
44	" — " (1901)	$\cdot 86 \pm \cdot 03$	" "	" " "
25	Swiss Cantons (Statistique de la Suisse)	$\cdot 71 \pm \cdot 07$	" "	" " "
143	Towns in Saxony (Ganz-Müller)	$\cdot 42 \pm \cdot 05$	" "	" " "

In Table VIII we collect the results yielded by data obtained from various sources. The figures of Würzburg are particularly interesting because, in his elaborate and careful paper, not only did he separate the rural from the urban districts, but he dealt with the whole of the German Empire and thus provided sufficient material for us to consider the nature of the regression of Infant Mortality upon the Birth Rate. Proceeding in the ordinary way, we have $r = \cdot 580$, $\eta = \cdot 593$ and $\frac{\sqrt{n}}{\cdot 67449} \times \frac{1}{2}\sqrt{\zeta} = 2\cdot 37$ (Pearson, Blakeman). Consequently the regression

¹ See on this Welton (Welton, 1911, p. 3).

may be regarded as effectively linear. This is of some importance, since our Bavarian material is not sufficiently extensive for us to be able to examine the regression in an altogether satisfactory way. The general impression which results from an examination of Table VIII is that our coefficient *may* be somewhat too large but that the difference from a reasonable expectation is not serious. Naturally, the coefficients in Table VIII measure total correlations, and do not bear comparison with our partial coefficients; this can be, *to some extent*, allowed for in the case of Heron's investigation, but we shall return to this shortly. In one set of observations which we have found, the poverty factor was experimentally eliminated. Thus Trap (cited by Saltet and Falkenburg, p. 15) published the result of an inquiry into the relation between size of family and the infant death rate, the parents being grouped under occupations. From these data (as given by Saltet and Falkenburg, p. 15) we obtained coefficients ranging from '6 to '9, but the material is not so presented as to admit of our determining the true probable errors, hence we do not feel justified in attaching importance to our deductions. It would have been possible to extend the figures in Table VIII, but sufficient have been given for our purpose; for a discussion of the relation between the birth rate and infant death rate, with other citations, reference should be made to Prinzing's treatise.

The Birth Rate and Poverty.

The statistical literature of this part of the subject is enormous and we shall observe the same limitations as before.

The most important contribution is Heron's monograph¹ (Heron, 1906). Heron dealt with the Metropolitan Boroughs and, as we have seen, founded the birth rate upon the number of wives aged 15-54 years. For his measure of poverty, Heron adopted several ingenious standards, the proportion of general labourers, the proportion of pawn-brokers, etc. All his coefficients are larger than ours but the difference is hardly material (Heron did not correct for populations in the way we have described but the effect of this does not seem great). Taking the result Heron obtained when he used the proportion of general labourers as a measure of poverty, it is to be remarked that the difference between his value and ours is less than its probable error ($\cdot 07 \pm \cdot 11$). Heron

¹ Niceforo (1911) has also studied the problem with the aid of the method of correlation, but the coefficient he used cannot be directly compared with those employed in this paper.

found that the correlation between the percentage of all kinds of pauperism and the birth rate was only $\cdot199 \pm \cdot145$; we ourselves have even obtained negative values in the case of English Registration Counties. On the other hand, the Bavarian returns yield coefficients not dissimilar from those of Heron when he employed a more direct measure of poverty than we have been able to use. In view of all the circumstances mentioned and the fact that Heron considered an urban, we a rural, population, the results may be taken to show that the percentage of *Unterstützte* is a fairly reliable measure of poverty for the purposes of an inquiry like the present.

TABLE IX.

Correlation between the Birth Rate and Various Indices of Poverty.

Source of data	Nature of Birth Rate	Measure of Poverty	Correlation	Computer
27 Metropolitan Boroughs (Heron (1))	% of married women aged 15-54	General Labourers per 1000	$\cdot52 \pm \cdot10$	Heron.
27 Metropolitan Boroughs (Heron (1))	% of married women aged 15-54	Pawnbrokers, etc., per 1000	$\cdot62 \pm \cdot08$	Heron.
27 Metropolitan Boroughs (Heron (1))	% of married women aged 15-54	Proportion living more than 2 in a room	$\cdot70 \pm \cdot07$	Heron.
27 Metropolitan Boroughs (Heron (1))	% of married women aged 15-54	Proportion of children aged 10-14 employed	$\cdot66 \pm \cdot07$	Heron.
142 Bavarian Rural Districts (Groth and Hahn)	% of all women aged 16-50	% of publicly assisted persons	$\cdot44 \pm \cdot05$	Greenwood & Brown.
22 Districts of Hamburg (Mombert)	% of married women aged 15-45	% of dwellings rented at less than 300 marks	$\cdot88 \pm \cdot03$	Greenwood & Brown.
26 Districts of Leipzig (Mombert)	% of all women aged 15-40	% of dwellings rented at less than 250 marks	$\cdot83 \pm \cdot04$	Greenwood & Brown.
26 Districts of Leipzig (Mombert)	% of all women aged 15-40	% of dwellings with less than 2 heated rooms	$\cdot91 \pm \cdot02$	Greenwood & Brown.

In Table IX we collect Heron's and others' results for comparison. The general conclusion seems to be that although our crude coefficient may be too small, it would be rash, for the purposes of subsequent calculation, to increase its value upon conjectural grounds.

Poverty and the Rate of Infant Mortality.

The association between poverty and infant mortality is also a mere commonplace of the literature; *inter alios* may be named—Malthus (1817), Neumann (1893), Verrijn Stuart (1901), Rowntree (1902), Heron (1906), Newman (1906), Prinzing (1906), Saltet and Falkenburg (1907), Local Government Board Report (1910). Few of these writers, however, give material for expressing the association in a quantitative form;

Heron's memoir is an exception. From his Table A (Heron, 1906, p. 6) we find the correlation between poverty (as measured by the proportion of general labourers) and the Infant Mortality Rate to be $\cdot40 \pm \cdot11^1$, a value which agrees admirably with our own findings. As we shall remark further on, it is doubtful how far this correlation really indicates an independent connection between the two variables, and we deeply regret being unable to submit our coefficient to other comparative tests. The conclusion to which our analysis tends on this point is, indeed, of considerable importance, *if true*; but we should like to have further warrant for believing it true than is afforded by a single comparison.

Artificial Feeding and the Rate of Infant Mortality.

Most of the authors already cited discuss this point; in addition we may refer to Creighton (1894, II. 766), Eröss (1903), The Sanitary Authority of Milan (1908), Seutemann (1909) and the Westminster Health Society (1910). In all the quantitative investigations we have found, the material is derived from a more or less complete census of the infant population of one city or borough, not from a series of districts as in Groth and Hahn's work. It was therefore necessary to adopt a

TABLE X.

Some Correlations between Survival and Breast-Feeding.

Source of Data	Correlation	Method	Notes
Barmen (Kriege and Seutemann, cited by Saltet and Falkenburg, p. 26*)	$\cdot41 \pm \cdot01$	Fourfold Table	The classification is into Breast- and Bottle-Fed children: correcting for income of Father raised r to $\cdot44 \pm \cdot01$.
Westminster (City of Westminster Health Soc.)	$\cdot30 \pm \cdot05$	„	† Partial Breast-Feeders included with full Breast-Feeders.
Derby (Howarth, cited by Newman, p. 238)	$\cdot33 \pm \cdot02$	„	Mixed Feeders included with Breast-Feeders.
Milan (La Mortalità infantile in Milano)	$\cdot29 \pm \cdot03$	„	Mixed Feeders included with Breast-Feeders.

* We have not found a copy of the Journal in which Kriege and Seutemann's paper appeared either at the British Museum or the two great Medical Libraries. There is a discrepancy between the figures as cited by Saltet and Falkenburg on p. 25 and p. 26 of their monograph. On p. 25, 4139 children are stated to have been alive on Aug. 15th, 1905. The table on p. 26 gives 4051. Possibly the difference is caused by omitting cases without particulars of Feeding. We have used the table on p. 26, *op. cit.*

† If the partial Breast-Feeders are included with the Bottle children, the correlation rises to $\cdot69$. A similar difference *but in the opposite direction* is produced in the Milan data by altering the classification.

¹ See p. 32.

different and hardly comparable method of measuring the correlation, the results of which appear in Table X. In our opinion, these coefficients are of but little use as comparative checks since, as noted in the table, the arithmetical value of the coefficient is greatly affected by the position of the dividing planes¹. The most that can be said, and said with hesitation, is that the coefficients agree fairly well among themselves and perhaps suggest that our value (obtained be it noted by a different method and with a different arrangement) may be too high. As will be pointed out later on, there is some internal evidence for this belief; but we should have liked to find stronger extrinsic confirmation of our view.

General result of the comparative Tests.

It may, we think, be asserted that such portions of our work as could be checked by comparison with the results of other investigators using the same method, or by reducing other material in a similar fashion, do not exhibit any startling contradictions. At the same time, we fully recognise the limitations which have been imposed upon this process by the nature of the subject. At the least, nothing has been elicited which would tend to discredit conclusions based upon Groth and Hahn's material. We shall now proceed to a closer scrutiny of our own constants.

IV. *The nature of the total regressions and of the frequency distributions for the Bavarian data.*

The precise value to be assigned to a determination of the coefficient of correlation depends upon the nature of the distribution of which it is a constant. While r is always some measure of association, the value deduced in any particular case can only be directly compared with the result of another investigation, if there are reasonable grounds for thinking that the correlation surfaces are approximately similar. A failure to recognise this fact has led untrained users of the method of correlation into difficulties. The coefficient of correlation attains its maximum utility when the regression of one character upon another is linear. Since, if the frequencies are normal, the regressions must necessarily be linear, this point was first examined. From the nature of the material, no values below 0 or above 100 can occur, so that the frequencies could not be truly Gaussian, although normal curves might

¹ Doubtless the frequencies are not normal.

give sufficiently close approximations to them. The principal data used are given in Appendix I and it is apparent upon examination that the distribution of percentage artificial feeding has no resemblance at all to a normal curve (see diagram). The other three variables conform to the type of "cocked-hat" distributions, but, on fitting normal curves and testing with Elderton's (1902) tables, no close agreement could be obtained. The least bad result was with the Poverty Rates ($P = .04$). Although the frequencies are not normal, it might still be that the regressions are linear and we have already found in the case of Würzburg's similar, but more extensive, data an example of effectively linear regression (Infant Death Rate upon Birth Rate). Owing to the relative sparsity of our data, the process employed in the case just referred to (Pearson, 1905; Blakeman, 1905) is not quite satisfactory, because the error introduced by grouping is considerable. For the purpose of determining η it was necessary to group the observations, but the value of r compared with it was deduced from the ungrouped figures. (The crude values, uncorrected for population, were used.)

TABLE XI.

Tests for Linearity of Regression (Crude Regressions).

Variables	r	η^*	$\frac{\sqrt{N}}{.67449} \times \frac{1}{2} \sqrt{\eta^2 - r^2}$
Birth Rate and Poverty Rate	.426	{ .513	{ 2.38
		{ .485	{ 2.06
Infant Death Rate and Poverty Rate	.465	{ .538	{ 2.41
		{ .534	{ 2.34
Infant Death Rate and Artificial Feeding Rate	.761	{ .785	{ 1.41
		{ .789	{ 1.51
Infant Death Rate and Birth Rate	.462	{ .746	{ 2.49
		{ .727	{ 1.95
Poverty Rate and Artificial Feeding Rate	.369	{ .490	{ 2.51
		{ .461	{ 1.71
Birth Rate and Artificial Feeding Rate	.462	{ .581	{ 2.23
		{ .521	{ 1.91

* The two values correspond to the two regressions, that of x upon y and of y upon x .

It will be noticed in Table XI that the values in the third column never sensibly exceed 2.5; this fact, together with information to be derived from plotting the original data on a diagram, seems to suggest that the regressions are effectively linear. The conclusion to be drawn from these results is that a regression equation of the first degree connecting the four variables should be of interest. In order to obtain the constants of such an equation it is necessary to determine the standard

deviations of the third order. As will be seen from the results collected in Appendix II, the means and standard deviations are somewhat affected by variations in the numbers of available pairs. For the present purpose we have adopted as the mean of each character that based upon all the available data and similarly the standard deviations of zero order were those deduced from the largest numbers of observations. We have not, in view of the relatively small effect on the coefficients of correlation which resulted from correcting for constant

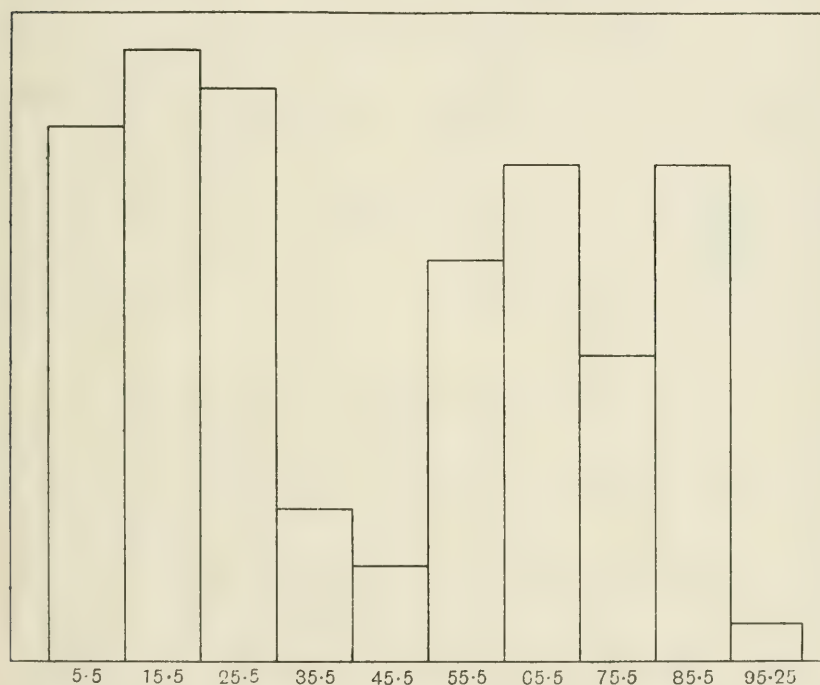


Fig. 1.

population, applied this process to the standard deviations. In determining the probable errors of the regression coefficients we have, as in the case of the partial correlation coefficients, given to n its smallest value. If x_1 be the rate of Infant Mortality, x_2 the Birth Rate (in terms of all women 16-50), x_3 the rate of Artificial Feeding and x_4 the Poverty Rate, then, measuring all rates from zero, we find :

$$x_1 = 1.234 x_2 + .141 x_3 + .936 x_4 - 4.334.$$

Comparing the coefficients of regression with their probable errors, we have

$$1.234 \pm .129, .141 \pm .012, .936 \pm .387.$$

Turning to the 84 districts with complete records and, in this case, correcting the Standard Deviations for population, we obtained the equation:

$$x_1 = 1.460 x_2 + .113 x_3 + 1.640 x_4 - 8.229.$$

The probable errors of the three regression coefficients are $\pm .111$, $\pm .011$, $\pm .358$. As will be seen, there is a considerable difference between the weights attaching to the x_4 term in the two equations. With regard to the two other variables on the right-hand side, it will be noticed that a unit change in x_2 would be associated with a larger change in x_1 than would a variation of the same extent in x_3 . This is, however, somewhat misleading from the practical standpoint since an absolute change of one per cent. in both the Birth Rate and the Artificial Feeding Rate would denote a much more substantial variation of the former than of the latter, the absolute values of their respective means being almost in the ratio of one to three. However, even if we allow for this fact, the Birth Rate term is of more weight than the Artificial Feeding term.

As will have been noticed in the diagram, the distribution of percentage artificial feeding tends to be bimodal, districts with percentages in the neighbourhood of fifty being in defect. This *may*, of course, indicate the real state of affairs. It may be that the mothers in one locality tend as a whole either to suckle their infants or to bring them up by hand. Fashion or some special economic conditions introducing heterogeneity might be responsible for such a result. On the other hand, it would not be right to neglect a different possibility. Groth and Hahn's data in this case are the result of circulating requests to the medical officers of the local vaccination departments (see p. 9) and they did not secure returns from anything like all the districts. It is just possible that when the medical officer found that either a very large or a very small proportion of the children brought under his notice were hand-fed, the circumstance attracted his attention and he did not fail to make a return; while in the instances in which the proportion of bottle-fed infants was only moderate, the information may have seemed commonplace and hardly worth recording. There is we imagine a somewhat general inclination to dwell upon extreme results in all inquiries, fostered by the tendency of writers to publish only the highest and lowest values of their census.

If anything of this kind has really occurred, the artificial feeding data are not a random sample but a selection. Pearson, in a classical memoir (1903), has shown that the value of a coefficient of correlation is much altered by selection. In this memoir, Pearson dealt with normal correlation and the case of the selected individuals being less variable than the population from which they were drawn, but has since indicated that the theorems then obtained were of greater generality than at first supposed. In the present case, the variability of the selection will be higher than that of the original population. In the case of normal correlation, it is easy to prove that a coefficient of correlation deduced by product moments from the selection will be larger than the value of r obtained by the same method from the original population. Illustrating this by an extreme case, let us suppose that part of the correlation surface which is included between parallel planes intersecting the surface at right angles to the horizontal plane and at distances $\pm h'$ from the centroid vertical has been removed. It will then be found that the coefficient of correlation deduced from what is left of the surface will be equal to $r \times \sqrt{\frac{1-p_2+2p_1}{1-p_2+2p_1r^2}}$ where r is the coefficient of the whole surface,

$$p_1 = \frac{h}{\sqrt{2\pi}} \times e^{-\frac{1}{2}h^2}, \quad p_2 = \frac{1}{\sqrt{2\pi}} \left(\int_{-h}^h e^{-\frac{x^2}{2}} dx \right), \quad h = \frac{h'}{\sigma_x}, \quad \sigma_x =$$

Standard Deviation of the "Selected" character.

For instance, if $h' = \sigma_x$ and $r = .5$, $p_1 = .2419$, $p_2 = .6827$, the apparent correlation is .676. A similar expression can be deduced when the frequency near the centroid is not removed but diminished¹.

Since we have no adequate means of determining how far the preliminary speculation may be just, it is unnecessary to enlarge upon the mathematical consequences which might flow from it. We desire, however, to emphasize the fact that if any such process of selection have really been at work, the true correlation between Artificial Feeding and Infant Mortality Rates is lower than would appear from our analyses. Further statistics are necessary to settle this important point² and we sincerely hope that an inquiry comparable with, but more extensive than that of Groth and Hahn³, may be embarked upon in our own country; evidently such a task is quite beyond the powers of private

¹ See Appendix III.

² Another possible source of bimodality which requires for its elucidation wider statistics is industrial differentiation.

³ Compare also the difference between the coefficients of Tables VII and VIIA.

individuals and could only be undertaken by the central government. In view of the very great and general interest which is aroused by the problems connected with infant mortality, such an inquiry ought not to be delayed, even although the amount of money and labour required were considerable.

V. The Meaning of the Partial Correlations.

Although the method of multiple correlation attains more nearly to the ideal of the scientific reasoner, who endeavours to isolate each member of a causal nexus, than do the ordinary processes of the official statistician, we must not over-estimate its powers. In particular, when the correlation is not normal, and in our case it is not normal, the partials represent in general no more than an average measure of association (*vid.* Yule, 1911, p. 248). We must also bear in mind the various objections to and limitations of our material which have been dwelt upon in the course of this paper.

At the same time, the results deduced seem of sufficient interest and importance for it to be worth while devoting some paragraphs to a discussion of their possible meaning. We hope that, in considering these speculations, the reader will bear in mind our previous criticisms and will understand that our conclusions are put forward mainly as suggestions which can be of service to future inquirers.

Infant Mortality and the Birth Rate.

The correlation between the Birth Rate and the Rate of Infant Mortality is not a new discovery and has, we think, been usually explained along the following lines. Those persons who marry young and do not restrain their procreative impulses tend to be drawn from a class of society in which the instinct of commercial prudence is not highly developed and the economic environment is bad. As the family increases in size, the environment becomes less and less satisfactory, with the growing number of mouths to feed. In other words, the association between the birth rate and the infant mortality rate is a consequence of the association between the former and poverty. Now, if our method had been completely adequate to the task of keeping the poverty factor constant, had our measure of economic status been thoroughly satisfactory, this current explanation would have to be modified in view of the results we have obtained. While we cannot pretend to have fully satisfied these conditions, enough has been done

to render it, in our judgment, probable that the association may be more direct than usually thought. A tempting speculation is suggested by the view entertained by the English eugenicists (see Schuster, 1905; Pearson, 1907 and 1909; Heron, 1907, and many other publications by Professor Pearson and his pupils) that stocks differentiated by physical or mental stigmata of inferiority are more fertile than the average. This phenomenon may, we think, be largely attributed to the employment of preventive checks by the superior artisan and middle class populations. It is not probable that successful or dominant types are naturally infertile; indeed it has been shown that longevity and fertility are correlated (Beeton, Pearson and Yule, 1901), while there is much evidence that longevity is an heritable character (Beeton and Pearson, 1899 and 1901). The relation between fertility and longevity was, however, determined on material probably little affected by artificial restrictions of fertility (see Beeton, Pearson and Yule, *op. cit.* pp. 160-1). The results just cited suggest that there is no inevitable association between fertility and inferiority and compel us to refer the fact—assuming it to be a fact—to the voluntary measures at present so much debated. The causation of the result does not, however, affect the argument based upon it. If the facts be as stated, it is easy to interpret our correlation. It can hardly be doubted that the offspring of pathologically weighted stocks will be, in general, less resistant to the ordinary dangers of childhood than members of normal families. The correlation between the birth rate and the infant death rate should be interpreted as a consequence of the association between the former and natural inferiority. A necessary inference would be that, under modern conditions, the birth rate is itself a better criterion of unfitness than any conventional measure of poverty such as the one here used. To avoid the risk of misconception, fostered by the pernicious habit of quoting detached phrases from a scientific paper, we once more emphasize the words *under modern conditions*. If those who are worthy citizens are relatively infertile, it is not in virtue of any immutable decree of fate but a consequence of conditions which may not have existed in the past (see Heron, 1906), are possibly not universal now and will *perhaps* disappear in the future. We are not discussing what must be but what is or—to be quite accurate—what may be. Another conclusion to be drawn from our present hypothesis is that the infant death rate must be, to some extent, selective; to what extent, is another question.

It is to be remarked that the whole of the previous argument falls to the ground if it be not a fact that the gross fertility of pathologically

weighted stocks is greater than the average. Pearson and Heron's results have been recently impugned by Weinberg (1911) but, as indicated in the footnote, this author's criticism appears to us to be based upon an arbitrary assumption¹.

At the same time, we are not prepared to deny that the relationship between fertility and mental or physical unfitness deserves further study.

Whatever may be thought of the speculation outlined above—we present it with the utmost diffidence—one conclusion appears highly probable. It is that the association between the actual Birth Rate and the Infant Death Rate is of considerable and direct importance², a phenomenon not lightly to be put on one side as of little immediate interest. We state this with some confidence, although a directly opposite opinion has received the sanction of so high an authority as Newsholme (1911, p. 44). Newsholme himself found a substantial correlation between the two rates (see p. 19) and we believe that, for the purpose in hand, the Bavarian data are more satisfactory than the returns from English Registration Counties. In working with the latter, it is impossible to avoid the difficulties introduced by mixing the populations of urban and rural districts. Other variables (for instance the effect of varying industrial conditions) are introduced into a problem already sufficiently complex, with the result that it becomes difficult to interpret the statistical constants deduced. It is needless to say that our conclusions on this as upon other points are subject to revision in the light of wider experience.

¹ Weinberg asserts that the difference in fertility between normal and pathological stocks found by Pearson, is spurious and due to the fertility of the normal stocks having been determined from the parental, that of the pathological stocks from the filial generation. He publishes (*op. cit.* p. 690) a so-called "General Proof" that the mean size of family based upon the "*Angaben*" of the children, will be greater than that deduced from a count of the parents. Obviously, if we have, say, two families, one with one, the other with two children, the mean size of family, based on the parental "*Angaben*" is 1.5, while if the children are questioned, the "*Angabe*"—"Two in Family"—will be given twice and "One in Family" once. From these replies, the mean family might be reckoned as 1.67. This appears to be all that Weinberg has succeeded in proving, and it seems to us unwarrantable in him to suppose that Pearson and Heron adopted so singular a method of reckoning. Evidently, if the names and pedigrees of the children are known—in the cases singled out for criticism by Weinberg they were known—no one could fall into this trap. We accordingly dissent from the conclusion stated by Weinberg at the foot of p. 690 (*op. cit.*) If Weinberg's "General Proof" has an interpretation other than that placed upon it in this note, we tender our apologies to him for having failed to discover it.

² See also the important discussion in Westergaard's treatise (p. 368).

Artificial feeding and the infant death rate.

Here again, in so far as we have succeeded in neutralising the factor of social environment, we have got rid of one obvious cause of infant mortality associated with artificial feeding, viz., the increased liability to septic contamination of the feeding bottles in poor households and the use of an inferior milk. But, apart from these accessory conditions, it is not surprising to find a positive association. Numerous experiments have demonstrated that the milk of each species is to some extent peculiar to it. After all, it does not seem likely that anything devised as a substitute for a normal secretion will on the average—neglecting the absolutely large but relatively small number of instances in which the natural secretion is demonstrably inadequate or of poor quality—vie with human milk as a food for infants. Further, it is not very far-fetched to suppose that all the reproductive functions are intimately correlated in a physiological sense, that diminished activity of the mammary glands may be associated with inferior viability of the offspring. It is, however, clear that the significant correlation between infant mortality and the prevalence of artificial feeding is the most hopeful result of our inquiry. While it may not be, and probably will not be, possible to arrive at a thoroughly efficient substitute for the method of breast feeding, it is undoubted that the progress of physiological chemistry and domestic hygiene will, step by step, enable us to approximate to the ideal. But, for reasons already given at length, we cannot think that the factor of artificial feeding is equal in importance to that measured by the Birth Rate.

Infant mortality and poverty.

Since we have, so far as our data permitted, eliminated the influence of the Birth Rate and Method of Feeding, it would be expected that the correlation might undergo some change, but the actual result in this, as well as the other correlations into which the factor of poverty enters, is at first sight perplexing. It is desirable to preface our discussion of results involving poverty with a few words of caution to the reader unversed in statistical methods. In interpreting the first regression equations, we laid no stress upon the term involving the poverty rate because the probable error of its constant was relatively large in one case. This was justifiable, but we should go beyond

our evidence were we to assert that a change in the poverty rate is definitely uncorrelated with a change in the rate of mortality. All we can say is, that, from the data before us, we have been unable to demonstrate that the given measure of poverty is closely associated with the Infant Mortality Rate (cf. the three partial coefficients involving the poverty rate with their probable errors as entered in Table VII)¹. A negative conclusion must not be transformed into a positive assertion. Let us now compare our partial coefficients with those to be deduced from Heron's (1906) memoir. Denoting the Birth Rate, the Artificial Feeding Rate, the Poverty Rate, and the Infant Death Rate by the subscripts, 1, 2, 3, 4, using Yule's notation for the partial coefficients and taking the Proportion of General Labourers as a measure of the Poverty Rate for Heron's data, we obtain the following comparative results.

	Heron	Greenwood & Brown
$r_{13\cdot4}$	$\cdot40 \pm \cdot11$	$\cdot14 \pm \cdot07$
$r_{13\cdot24}$	—	$\cdot14 \pm \cdot07$
$r_{14\cdot3}$	$\cdot37 \pm \cdot11$	$\cdot64 \pm \cdot04$
$r_{14\cdot23}$	—	$\cdot57 \pm \cdot05$
$r_{34\cdot1}$	$\cdot19 \pm \cdot13$	$\cdot28 \pm \cdot07$
$r_{34\cdot12}$	—	$\cdot17 \pm \cdot07$

If we compare our second order coefficients with the first order coefficients of Heron's work (his data did not enable him to deal with a fourth variable), we find the differences are, $+\cdot26 \pm \cdot13$, $-\cdot20 \pm \cdot12$, $\cdot02 \pm \cdot15$. While it cannot be said that any one of these differences is necessarily significant, it is worthy of remark that our Birth Rate-Poverty Rate coefficient is smaller, our Birth Rate-Infant Death Rate coefficient larger than Heron's. We are tempted to explain the second finding by the inferiority of our method of measuring the Birth Rate; and we have little doubt that his more satisfactory measure of poverty has been influential in the first case. Still, when all allowances are made, we should be disposed to attribute greater importance to the fact that we are not only dealing with another country, but also with a different class of population. Heron pointed out—and it is not the least interesting part of a valuable paper—that the intensities of some of the correlations he found were very different at different epochs². Another point to be noticed is that he obtained a higher correlation

¹ Note also the relatively and absolutely greater weight attaching to the poverty term in the equation deduced from the 84 districts of Table VII A.

² In the London Boroughs, the correlation between Infant Mortality Rate and Birth Rate was *negative* in 1851, $-\cdot30 \pm \cdot10$, positive in 1901, $+\cdot51 \pm \cdot10$. (Heron, 1906, p. 18.)

between the Poverty Rate and the Birth Rate when the proportion of pawnbrokers was used as the measure of poverty than when the proportion of general labourers was chosen, although the difference may not be significant. The suggestion immediately arises as to whether the sinister associations of poverty in urban districts may really be, not functions of pure poverty, regarded as an environmental condition, but of a peculiar type of organisation.

In life as we know it in a modern city, the economic struggle for existence is so keen, the penalty of failure so heavy, that the weakest must be forced to the wall. Whether success in this struggle be the highest kind of success is an irrelevant consideration; at least all those who, under any definition of a question-begging term, are "fit" will endeavour not to fail. It accordingly results that, in towns, economic success is, to some extent, a measure of civic fitness. In the case of rural districts, however, the economic test, although still a rough approximation to the truth, may be definitely less adequate than in industrial towns. Were the preceding remarks substantiated, our results would be explained, but we have no evidence before us of a character sufficiently definite to justify further speculations¹.

The Birth Rate and the Artificial Feeding Rate.

As previously remarked (p. 10), Groth and Hahn concluded that prolonged lactation tended markedly to lower the Birth Rate, a somewhat wide-spread belief in certain classes. The fact that the total correlation between the Artificial Feeding and Birth Rates is positive would tend to support the conclusion, but the effect of neutralising other factors by the method of partial correlation is such that we are inclined to doubt its truth. If our view be correct, this case shows the importance of employing the language of multiple correlation in problems such as the one before us.

VI. Conclusions respecting the chief factors of Infant Mortality.

We have now described to the best of our ability both the good and bad points of the material collected by Groth and Hahn, together with the methods we adopted in analysing it. With respect to Infant Mortality in the rural districts of Bavaria, we conclude:

(1) Of the factors measured by Groth and Hahn the most important is the Birth Rate. A high Birth Rate tends to be associated

¹ It is impossible to pass from speculations to well-weighted conclusions until we have a stricter definition and better criterion of poverty than at present available.

with a high Infant Death Rate and this association cannot be explained by any inter-relations between either variable and proportional poverty or artificial feeding.

(2) It follows, as a corollary to (1), that a considerable share in the causation of infant mortality should be attributed to a factor beyond the ordinary sphere of preventive medicine.

(3) The habit of artificially feeding infants is definitely correlated with their rate of mortality, so that some improvement in the latter may be reasonably expected with the growth of physiological knowledge and public hygiene.

(4) It has not been possible to demonstrate any unambiguous association between poverty, as measured by Groth and Hahn, and the death rate of infants.

(5) The same remark applies to the relation between breast feeding and the Birth Rate, as in the case of Poverty and the Infant Death Rate.

VII. *The selective value of a high rate of Infant Mortality.*

As mentioned in the introduction to this paper, much attention has been devoted to the supposed selective importance of a high rate of infant mortality. The two most recent investigators are Yule (Local Gov. Board, 1910, Appendix I) and Snow (1911) and their results are, to some extent, discordant. The difficulty of the problem is very considerable. The fact that a high rate of infant mortality is usually associated with a high death rate at all ages, does not prove the non-selective character of the former, since the influence of an unfavourable environment might well act in the same sense at all ages. The efforts of both workers have accordingly been directed to discover an adequate measure of this environment and then, having neutralised it by the method of multiple correlation, to measure the partial correlation between the mortalities of infancy and later ages. We have grave doubts as to whether the problem can be solved with the help of mortality statistics alone and we are unable to share the confidence with which Snow appears to regard certain of his conclusions. It is not, however, necessary for us to enter upon a critical examination of a subject of great intricacy since, although Groth and Hahn touch upon the point, they do not provide data of an altogether satisfactory kind. What they deal with is the relation between Infant Mortality, the percentage of approved recruits, the prevalence of Artificial Feeding and the Death Rate of young adults. Unfortunately they do not bring

into relation the returns of Infant Mortality for, say, 1880 and the recruiting statistics for 1900.

While it is quite likely that inter-local differences at any one time are much greater than the changes within any one district over a small number of years, we can have but little confidence in the data for the present object. On these grounds, we have only calculated one set of constants, taking as variables, Infant Mortality, Artificial Feeding, Mortality at ages 11-20 and the Fitness of Recruits.

TABLE XII.

Correlation between Infant Mortality, Death Rate (11-20 years), Percentage Fitness of Recruits and Artificial Feeding, Bavarian Rural Districts.

Variables	Observations	Correlation
Death Rate (11-20) and Infant Mortality Rate ...	92	$\cdot084 \pm \cdot070$
Infant Mort. Rate and Percentage Fitness of Recruits	92	$\cdot125 \pm \cdot069$
Death Rate (11-20) and Percentage Fitness of Recruits	92	$-\cdot195 \pm \cdot068$
Death Rate (11-20) and Artificial Feeding Rate ...	92	$\cdot159 \pm \cdot069$
Art. Feeding Rate and Infant Mort. Rate ...	92	$\cdot755 \pm \cdot030$
Art. Feeding Rate and Percentage Fitness of Recruits	92	$-\cdot054 \pm \cdot070$

Correlation between each of the Variables and Population.

Death Rate (11-20) and Population ...	$-\cdot072 \pm \cdot070$
Infant Mortality and Population ...	$-\cdot061 \pm \cdot070$
Percentage Fitness of Recruits and Population ...	$-\cdot055 \pm \cdot070$
Artificial Feeding and Population ...	$-\cdot055 \pm \cdot070$

Total Correlation with Population Constant.

Death Rate (11-20) and Infant Mortality Rate ...	$\cdot080 \pm \cdot070$
Infant Mortality Rate and Percentage Fitness of Recruits	$\cdot122 \pm \cdot069$
Death Rate (11-20) and Percentage Fitness of Recruits ...	$-\cdot200 \pm \cdot068$
Death Rate (11-20) and Artificial Feeding Rate ...	$\cdot155 \pm \cdot069$
Artificial Feeding Rate and Infant Mortality Rate ...	$\cdot754 \pm \cdot030$
Artificial Feeding Rate and Percentage Fitness of Recruits	$\cdot057 \pm \cdot070$

Partial Correlations with Population Constant.

Variables	Constants	Correlation
Death Rate (11-20) and Inf. Mort. Rate	Percentage Fitness of Recruits and Art. Feeding Rate	$-\cdot009 \pm \cdot070$
Inf. Mort. Rate and Percentage Fitness of Recruits	Death Rate (11-20) and Art. Feeding Rate	$\cdot245 \pm \cdot066$
Death Rate (11-20) and Percentage Fitness of Recruits	Inf. Mort. Rate and Art. Feeding Rate	$-\cdot186 \pm \cdot068$
Art. Feeding Rate and Death Rate (11-20)	Inf. Mort. Rate and Percentage Fitness of Recruits	$\cdot102 \pm \cdot070$
Art. Feeding Rate and Inf. Mort. Rate	Death Rate (11-20) and Percentage Fitness of Recruits	$\cdot765 \pm \cdot029$
Art. Feeding Rate and Percentage Fitness of Recruits	Death Rate (11-20) and Inf. Mort. Rate	$-\cdot204 \pm \cdot067$

The results appear in Table XII¹.

On account of the objections already mentioned, we do not propose to discuss the table at length. Apart from the Artificial Feeding-Infant Mortality correlation, already considered in detail, only two of the coefficients exceed thrice the probable error. If the percentage fitness of recruits be accepted as a measure of fitness in the true sense—as to which we have grave doubts—then the result affords some evidence of the selective value of an infant death rate. If, on the other hand, we regard the recruiting standard as a measure of environment and the death rate at ages 11–20 as a test of fitness, no statement can be made. For our own part, we should hesitate to draw any definite conclusions whatever.

CONCLUDING REMARKS.

Any impartial reader of this memoir will, we think, be apt to form some such conclusion as the following.

Since we have thought it desirable to devote a good deal of labour to the reduction of Groth and Hahn's material, we are not likely to have underestimated its value. Since, in the face of so natural a prepossession in favour of the evidence, we have been compelled again and again to emphasize the difficulty of basing trustworthy conclusions upon our analytical results, since, lastly, these results conflict with opinions often and confidently expressed, our investigation may be regarded as of no value.

We are far from suggesting that such an opinion is without foundation, but we believe some important submissions can be made in arrest of judgment. We would inquire whether the statements upon which popular and current medical opinions depend do really deserve more credence than the data here examined; whether much that passes for evidence is really more than optimistic dogma.

A knowledge of the factors controlling the rate of infant mortality can, we believe, only be attained by the methods of statistical science, indeed only by a refined handling of those methods. The day is far distant when the problem can safely be discussed by the proverbial "man in the street." We are not, therefore, without hope that the present memoir, in spite of—perhaps, because of—its tentative and incomplete character, will be of service to fellow-labourers.

¹ For the purposes of this table only those data were used which recorded all four variables. This accounts for the slight discrepancy between the value of the Artificial Feeding-Infant Mortality coefficient of this table and of those given previously.

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APPENDIX I.

Principal Data analysed in the text (Columns (2) and (4) are from unpublished data supplied by the Bavarian Statistical Office, the remainder are from Groth and Hahn's work).

Name of District	1 Population aver- age, 1900-4	2 Number of wo- men aged 16-50, Census 1901	3 Crude Birth Rate, 1900-4	4 Birth Rate in terms of Column 2	5 Poverty Rate, 1899-1902	6 Infant Death Rate, 1900-4	7 Percentage of children not Breast-fed, 1903-8	8 Number of child- ren upon which (7) is based
Oberbayern :								
Aibling ...	22512	5093	3·46	15·27	2·51	24·6	63·5	1159
Aichach ...	27437	6115	4·26	19·09	2·80	33·6	63·3	770
Altötting ...	35177	8044	3·35	14·65	3·15	25·6	83·8	1122
Berchtesgaden ...	21512	5172	3·18	13·21	3·98	20·0	82·4	437
Bruck ...	26013	5440	4·17	19·94	1·51	38·6	—	—
Dachau ...	26572	5841	4·40	20·01	1·80	34·7	70·7	792
Ebersberg ...	25953	5569	3·81	17·77	2·18	35·3	—	—
Erding ...	41944	8953	3·88	18·18	3·09	34·8	—	—
Freising...	33369	7475	4·54	20·26	2·98	34·3	71·8	501
Friedberg ...	34425	8041	4·73	20·23	2·68	39·7	68·0	1053
Garmisch ...	13496	3072	3·15	13·85	1·47	21·0	—	—
Ingolstadt ...	24627	5227	5·08	23·94	2·38	40·7	—	—
Landsberg ...	24828	5290	3·85	18·06	1·80	28·3	—	—
Laufen ...	32954	7289	3·29	14·89	2·49	22·1	68·5	833
Miesbach ...	34834	7683	3·54	16·07	1·32	22·0	84·2	228
Mühldorf ...	37968	8401	3·75	16·93	2·35	29·8	87·2	476
München I ..	44092	10214	4·40	19·00	1·49	38·4	75·4	999
München II :								
†Starnberg ...	18782	7924	3·60	15·26	1·91	25·6	—	—
Wolfratshausen...	17166		3·11		2·73	26·7	90·1	462
Pfaffenhofen ...	35599	7468	4·58	21·83	2·95	38·5	70·2	991
Rosenheim ...	38248	8541	3·44	15·40	1·84	21·6	55·0	1545
Schongau ...	20453	4384	3·56	16·60	1·89	24·7	—	—
Schrobenhausen ...	20414	4498	4·52	20·52	2·99	35·7	58·8	583
Tölz ...	16834	3898	2·98	12·87	2·83	22·2	77·0	400
Traunstein ...	41589	9316	3·39	15·11	1·92	22·1	69·8	696
Wasserburg ...	36908	7939	3·38	15·69	3·12	27·8	56·4	553
Weilheim ...	31637	6960	3·58	16·27	2·17	22·2	—	—
Niederbayern :								
Bogen ...	31631	6824	4·29	19·88	4·18	33·4	70·8	364
Deggendorf ...	38274	8228	3·99	18·56	4·00	32·3	—	—
Dingolfing ...	22260	5078	3·86	16·91	3·65	33·9	86·7	581
Eggenfelden ...	37419	8244	3·88	17·59	2·09	32·2	91·9	333
Grafenau ...	18885	3928	4·37	21·02	5·43	26·2	—	—
Griesbach ...	33756	7353	3·75	17·23	3·08	28·4	82·8	1224
Kelheim ...	33794	7422	4·52	20·57	3·34	41·2	—	—
Kötzting ...	25787	5505	4·53	21·21	3·87	25·6	—	—
Landau a. I. ...	22995	4992	3·98	18·33	3·46	31·4	—	—
Landshut ...	29216	6409	4·23	19·28	3·01	33·7	—	—

† The number of women aged 16-50 only obtained for the joint district in these cases.

Appendix I, Principal Data etc. (continued).

Name of District	1 Population aver- age 1900—4	2 Number of wo- men aged 16—50, Census 1901	3 Crude Birth Rate, 1900—4	4 Birth Rate in terms of Column 2	5 Poverty Rate, 1899—1902	6 Infant Death Rate, 1900—4	7 Percentage of children not breast-fed, 1903—8	8 Number of child- ren upon which (7) is based
Niederbayern (cont.):								
Mallersdorf ...	23529	5357	4·24	18·61	3·37	36·2	77·4	1297
Passau ...	42367	9381	3·76	16·98	3·65	29·4	—	—
Pfarrkirchen ...	36666	8318	3·75	16·52	2·86	30·3	90·4	466
Regen ...	27603	5883	4·57	21·44	3·45	29·6	39·1	884
†Rottenburg ...	18084	7546	4·35	20·76	3·92	34·1	80·8	468
Mainburg ...	16735		4·67		2·99	37·2	75·9	552
Straubing ...	22255	5097	3·92	17·13	2·90	35·2	83·8	1156
Viechtach ...	22492	4868	4·27	19·75	3·49	27·3	—	—
Vilsbiburg ...	30733	6906	3·80	16·91	2·50	31·8	—	—
Vilshofen ...	42948	9389	3·96	18·11	4·61	31·9	—	—
Wegscheid ...	17268	3722	3·67	17·05	4·43	23·8	—	—
Wolfstein ...	30136	6402	4·00	18·81	3·12	27·2	50·5	376
*Pfalz (Bavarian Palatinate):								
Bergzabern ...	38683	8776	3·15	13·87	—	14·6	16·4	456
†Dürkheim ...	28954	19475	2·75	13·16	—	16·6	28·1	595
Neustadt a. H. ...	51263		3·45		—	16·7	12·5	939
Frankenthal ...	62563	14256	3·67	16·12	—	18·1	24·8	749
Germersheim ...	53979	12098	3·36	14·99	—	19·3	—	—
Homburg ...	65052	14015	4·18	19·42	—	13·3	—	—
Kaiserslautern ...	84975	19913	3·73	15·90	—	14·2	15·0	1429
Kirchheimbolanden	26238	6035	3·22	13·99	—	16·3	16·4	664
Kusel ...	44718	9860	3·45	15·65	—	10·9	12·1	347
Landau ...	70769	16655	2·95	12·55	—	17·5	—	—
Ludwigshafena. Rh.	97010	21725	4·65	21·77	—	22·2	—	—
Pirmasens ...	74531	16451	4·27	19·34	—	17·3	6·8	427
Rockenhausen ...	38685	8831	3·14	12·57	—	12·0	6·8	250
Speyer ...	39307	9652	3·81	15·53	—	25·0	—	—
†St Ingbert ...	38459	17977	4·40	17·72	—	13·7	6·1	668
Zweibrücken ...	43289		3·44		—	14·5	—	—
Oberpfalz:								
Amberg ...	25679	5893	4·17	18·16	1·81	29·3	—	—
Beilngries ...	28853	6536	4·19	18·48	2·74	38·7	—	—
Burglengenfeld ...	27312	5934	4·49	20·65	2·82	35·5	—	—
Cham ...	28925	6058	4·38	20·93	2·96	27·3	25·0	372
Eschenbach ...	22349	5150	3·32	14·42	2·36	18·4	—	—
Kemnath ...	22901	4820	3·62	17·19	2·58	15·5	—	—
Nabburg ...	17948	4068	3·92	17·29	3·45	26·0	23·4	516
Neumarkt ...	28668	7357	3·79	14·78	3·14	33·6	25·4	421
Neunburg v. W. ...	15119	3289	3·96	18·21	1·73	27·5	—	—
Neustadt a. W.-N.	36620	7980	3·82	17·55	1·78	19·7	—	—
Oberviechtach ...	15648	3293	3·94	18·69	1·24	25·8	—	—
Parsberg ...	28953	6825	4·56	19·36	2·45	40·1	—	—
Regensburg ...	29993	6665	4·36	19·63	3·98	37·6	81·8	967

* No particulars of Poverty Rate are available for the Bavarian Palatinate.

Appendix I, Principal Data etc. (continued).

Name of District	1 Population aver- age, 1900—4	2 Number of wo- men aged 16—50, Census 1901	3 Crude Birth Rate, 1900—4	4 Birth Rate in terms of Column 2	5 Poverty Rate, 1899—1902	6 Infant Death Rate, 1900—4	7 Percentage of children not Breast-fed, 1903—8	8 Number of child- ren upon which (7) is based
Oberpfalz (cont.):								
Roding ...	23825	5194	4.14	18.99	3.93	30.4	54.3	313
Stadtamhof ...	42280	9690	5.01	21.87	3.64	40.4	56.9	810
Sulzbach ...	20338	4723	3.56	15.31	2.66	23.4	—	—
Tirschenreuth ...	35366	7717	3.47	15.90	2.41	18.1	—	—
Vohenstrauss ...	23787	5111	3.87	18.02	2.56	20.9	—	—
Waldmünchen ...	15831	3377	3.98	18.68	3.64	23.6	15.4	479
Oberfranken:								
Bamberg I ...	25347	5964	3.33	14.16	1.39	21.8	25.4	335
Bamberg II ...	28973	6433	3.56	16.04	1.55	23.0	11.5	286
Bayreuth ...	27700	6347	3.44	15.02	2.38	14.4	15.5	824
Berneck... ...	15271	3379	3.08	13.92	2.80	13.1	8.4	428
Ebermannstadt ...	22353	5253	3.03	12.89	1.86	15.8	9.4	595
Forchheim ...	28508	6655	3.18	13.63	1.60	17.1	—	—
Höchstädt a. A. ...	27516	6365	3.62	15.64	2.09	22.2	—	—
Hof ...	25962	5716	3.40	15.43	1.85	13.7	9.3	1389
Kronach ...	31684	7103	3.89	17.35	2.29	19.6	—	—
Kulmbach ...	25911	6003	3.11	13.44	1.72	13.2	11.3	785
Lichtenfels ...	33407	7901	3.55	15.01	1.58	21.2	—	—
Münchberg ...	27619	5982	3.07	14.17	2.08	12.0	4.8	769
Naila ...	22940	4910	3.22	15.04	1.32	13.2	10.0	619
Pegnitz ...	26456	6109	3.25	14.05	2.43	15.7	7.4	719
Rehau ...	25392	5442	3.26	15.19	1.62	14.8	—	—
Stadtsteinach ...	17252	3778	3.25	14.85	2.28	15.5	4.5	200
Staffelstein ...	18932	4569	3.02	12.51	1.82	20.6	22.6	336
Teuschnitz ...	18670	4150	3.87	17.42	0.86	19.9	5.2	419
Wunsiedel ...	45433	9757	3.40	15.81	1.97	13.7	11.6	595
Mittelfranken:								
Ansbach ...	33295	7894	3.09	13.02	1.72	20.4	18.6	307
Dinkelsbühl ...	24103	5726	3.28	13.81	2.14	28.3	—	—
Eichstätt ...	23752	5006	4.58	21.71	2.46	37.9	83.8	730
Erlangen ...	13221	3086	3.53	15.13	2.34	21.8	—	—
Feuchtwangen ...	26057	6064	3.36	14.45	3.05	26.0	23.0	665
Fürth ...	28397	6706	3.99	16.88	2.29	26.6	50.6	160
Gunzenhausen ...	31938	7520	3.22	13.66	2.81	24.4	39.5	456
Hersbruck ...	41742	9283	3.32	14.94	2.53	21.5	—	—
Hilpoltstein ...	23550	5364	3.66	16.07	3.01	31.3	—	—
Neustadt a. A. ...	30044	6947	3.17	13.72	1.94	19.1	22.0	700
Nürnberg ...	20991	4703	3.93	17.55	1.89	26.7	45.6	193
Rothenburg a. T....	19623	4653	2.73	11.52	2.81	15.9	19.6	225
Scheinfeld ...	18935	4501	2.77	11.65	2.37	17.6	—	—
Schwabach ...	34270	8038	4.00	17.06	2.71	30.5	36.7	479
Uffenheim ...	30250	7267	2.55	10.67	2.62	17.6	37.8	579
Weissenburg i. B....	28012	6463	3.51	15.23	2.89	28.4	54.0	309

Appendix I, Principal Data etc. (continued).

Name of District	1 Population aver- age, 1900—4	2 Number of wo- men aged 16—50, Census 1901	3 Crude Birth Rate, 1900—4	4 Birth Rate in terms of Column 2	5 Poverty Rate, 1899—1902	6 Infant Death Rate, 1900—4	7 Percentage of children not Breast-fed, 1903—8	8 Number of child- ren upon which (7) is based
Unterfranken :								
Alzenau ...	22299	4908	3·84	17·45	0·89	14·0	—	—
Aschaffenburg ...	32071	7927	4·25	17·21	1·66	16·2	8·1	2048
Brückenau ...	12870	2840	3·27	14·80	1·38	13·7	21·1	346
Ebern ...	18900	4256	3·36	14·92	1·46	18·8	—	—
Gerolzhofen ...	30693	7112	3·16	13·64	2·81	19·0	16·6	313
Hammelburg ...	19631	4415	3·19	14·19	1·86	16·2	8·2	281
Hassfurt ...	28309	6479	3·82	16·69	2·07	21·9	29·5	536
Hofheim ...	13818	3310	3·00	12·52	0·88	18·0	24·9	329
Karlstadt ...	30199	7069	3·29	14·06	1·90	20·9	—	—
Kissingen ...	34473	8310	3·45	14·32	2·05	16·8	20·7	421
Kitzingen ..	29857	6985	2·78	11·89	2·68	17·7	17·7	265
Königshofen ...	14758	3498	3·11	13·12	1·56	19·3	—	—
†Lohr ...	19773	7995	3·74	15·62	2·08	20·5	17·3	1256
Gemünden ...	14853		3·42		1·95	16·2	16·2	401
Marktheidenfeld ...	30847	7061	3·37	14·71	1·83	17·9	—	—
Mellrichstadt ...	13426	3109	2·99	12·92	1·68	12·3	—	—
Miltenberg ...	21721	4832	3·37	15·16	1·59	16·7	—	—
Neustadt a. S. ...	19805	4620	3·35	14·38	2·69	17·4	—	—
Obernburg ...	27271	6045	3·35	15·09	1·57	14·4	3·7	540
Ochsenfurt ...	26625	6353	2·90	12·15	1·90	21·4	—	—
Schweinfurt ...	34703	8199	3·30	13·95	1·39	18·8	20·9	253
Würzburg ...	41728	9824	3·54	15·02	1·89	25·2	23·2	1008
Schwaben :								
Augsburg ...	40557	9507	4·17	17·80	2·22	34·3	68·6	1139
Dillingen ...	37507	8029	3·74	17·49	3·02	30·7	81·4	778
Donauwörth ...	32610	6582	3·76	18·61	2·23	33·1	—	—
Füssen ...	18805	4223	3·43	15·29	1·59	21·1	58·0	336
Günzburg ...	29905	6620	3·92	17·71	2·53	32·0	—	—
Illertissen ...	19606	4155	3·77	17·77	2·30	25·2	67·6	562
Kaufbeuren ...	23684	5142	3·61	16·61	1·57	26·1	—	—
Kempten ...	34397	7715	3·35	14·92	1·45	25·1	—	—
Krumbach ...	23849	5513	3·53	15·28	2·10	24·1	—	—
Lindau ...	31162	6936	3·05	13·71	1·40	18·6	—	—
Memmingen ...	30754	6761	3·46	15·76	1·33	23·2	71·1	422
Mindelheim ...	34094	7626	3·55	15·87	1·44	23·8	59·2	508
Neuburg a. D. ...	29319	6262	4·31	20·19	3·50	33·9	60·7	900
Neu-Ulm ...	20634	4562	3·59	16·25	2·05	24·8	46·3	227
Nördlingen ...	30675	7090	3·38	14·61	2·34	26·5	—	—
Oberdorf ...	24195	5233	3·19	14·74	1·14	22·1	65·4	680
Schwabmünchen ...	22305	4750	3·93	18·44	2·52	29·3	69·3	593
Sonthofen ...	34270	8235	2·96	12·33	1·32	18·9	57·7	307
Wertingen ...	18560	3936	3·91	18·47	2·55	29·1	65·9	1191
Zusmarshausen ...	16024	3437	3·95	18·42	2·13	27·2	65·8	433

APPENDIX II.

Table to show how the Means and Standard Deviations are affected by Variations in the numbers of Pairs available for different Correlations.

Character			Mean	Standard Deviation	Number of Observations
Infant	Mortality	Rate	24·309	7·642	161
”	”	”	24·379	7·617	156
”	”	”	25·183	7·468	145
”	”	”	23·956 [24·917]	7·839 [7·592]	97
Birth	Rate	...	16·429	2·589	156
”	”	...	16·481	2·574	142
”	”	...	16·225 [16·334]	2·616 [2·640]	92
Poverty	Rate	...	2·408	·821	145
”	”	...	2·404	·821	142
”	”	...	2·427 [2·417]	·775 [·776]	87
Artificial	Feeding	Rate	43·367	28·355	97
”	”	”	43·404	27·951	92
”	”	”	46·685 [46·148]	27·999 [27·678]	87

[The values in brackets are those for 84 districts with records of each variable.]

APPENDIX III.

The Effect on the Coefficient of Correlation of excluding a Proportion of the Frequency near the Mode of One Character.

Let the original correlation surface be normal, *i.e.*, let:

$$z = z_0 e^{-\frac{1}{2}(1-r^2)\left(\frac{x^2}{\sigma_1^2} - \frac{2rxy}{\sigma_1\sigma_2} + \frac{y^2}{\sigma_2^2}\right)}$$

where σ_1 and σ_2 are the standard deviations of the x and y characters and r the coefficient of correlation.

If z be multiplied by $\left(k - e^{-\frac{x^2}{2c^2}}\right)$ where k and c are constants, the former not less than unity, we shall be practising a method of exclusion which becomes more and more stringent as we approach the centroid. The coefficient of correlation deduced by product moments from this selected surface is given by the equation:

$$r_1 = \frac{\int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} kxxy \, dx \, dy - \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} e^{-\frac{x^2}{2c^2}} xxy \, dx \, dy}{\sqrt{\left\{ \left(\int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} kx^2z \, dx \, dy - \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} e^{-\frac{x^2}{2c^2}} x^2z \, dx \, dy \right) \left(\int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} ky^2z \, dx \, dy - \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} e^{-\frac{x^2}{2c^2}} y^2z \, dx \, dy \right) \right\}}}$$

we easily find:

$$\begin{aligned} \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} e^{-\frac{x^2}{2c^2}} xyz \, dx \, dy &= \frac{N\sigma_1\sigma_2rc^3}{(\sigma_1^2 + c^2)^{\frac{3}{2}}} \\ \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} e^{-\frac{x^2}{2c^2}} x^2z \, dx \, dy &= \frac{N\sigma_1^2c^3}{(\sigma_1^2 + c^2)^{\frac{3}{2}}} \\ \int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} e^{-\frac{x^2}{2c^2}} y^2z \, dx \, dy &= \frac{N\sigma_2^2c(\sigma_1^2 + c^2 - r^2\sigma_1^2)}{(\sigma_1^2 + c^2)^{\frac{3}{2}}} \end{aligned}$$

The other three integrals are, of course, $kN\sigma_1\sigma_2r$, $kN\sigma_1^2$ and $kN\sigma_2^2$ respectively and we find on substituting:

$$r_1 = r \times \sqrt{\frac{k(\sigma_1^2 + c^2)^{\frac{3}{2}} - c^3}{k(\sigma_1^2 + c^2)^{\frac{3}{2}} - c^3 - \sigma_1^2c(1 - r^2)}} \dots\dots\dots(1)$$

i.e., r_1 is numerically greater than r for all values of the latter between 0 and ± 1 .

My friend Mr G. Udny Yule, with whom I have discussed the problem referred to in this note, has pointed out to me that a result identical with (1) can be deduced from Pearson's (1903) constants for a selected population obeying the normal law. The distribution here

considered will be the original population of Pearson's case less the normal selection (*e.g.*, if the selected individuals represent survivors the distribution will be that of deaths). Thus, Mr Yule finds:

“SELECTION.”

“REST.”

$$N' = N \times \frac{yc}{\sqrt{c^2 + \sigma_1^2}}$$

$$N'' = N \left(1 - \frac{yc}{\sqrt{c^2 + \sigma_1^2}} \right)$$

$$S_1 = \sigma_1 \times \frac{c}{\sqrt{c^2 + \sigma_1^2}}$$

$$S_1'^2 = \frac{\sigma_1^2}{c^2 + \sigma_1^2} \times \frac{(c^2 + \sigma_1^2)^{\frac{3}{2}} - yc^3}{\sqrt{(c^2 + \sigma_1^2)} - yc} \quad [\text{since } N' S_1'^2 + N'' S_1''^2 = N \sigma_1^2]$$

$$S_2 = \sigma_2 \times \frac{\sqrt{c^2 + (1-r^2)\sigma_1^2}}{\sqrt{c^2 + \sigma_1^2}}$$

$$S_2'^2 = \frac{\sigma_2^2}{c^2 + \sigma_1^2} \times \frac{(c^2 + \sigma_1^2)^{\frac{3}{2}} - yc^3 - yc\sigma_1^2(1-r^2)}{\sqrt{(c^2 + \sigma_1^2)} - yc}$$

$$p' = R' s_1 s_2 = p \times \frac{c^2}{c^2 + \sigma_1^2}$$

$$p'' = \frac{p}{c^2 + \sigma_1^2} \times \frac{(c^2 + \sigma_1^2)^{\frac{3}{2}} - yc^3}{\sqrt{(c^2 + \sigma_1^2)} - yc}$$

$$R' = r \times \frac{c}{\sqrt{c^2 + (1-r^2)\sigma_1^2}}$$

$$R'' = r \times \sqrt{\frac{(c^2 + \sigma_1^2)^{\frac{3}{2}} - yc^3}{(c^2 + \sigma_1^2)^{\frac{3}{2}} - yc^3 - yc\sigma_1^2(1-r^2)}}$$

where σ , s and S' represent the standard deviations, N , N' and N'' the populations, r , R' and R'' the coefficients of correlation, p , p' and p'' the product moments of the whole population, “Selection” and “Rest” respectively, while y is a constant less than unity.

It will be seen that Mr Yule's R'' agrees with (1) *supra* when we substitute $\frac{1}{y}$ for k in the latter.

The point is a simple one, but I do not remember to have seen it dealt with elsewhere.

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THE DANGERS OF SALINE INJECTIONS. BY
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(From the Lister Institute of Preventive Medicine.)

IN recent years the practice of injecting hot solutions of saline into a vein, or under the skin, has greatly increased. The use of these injections in the prevention or treatment of operative shock, in the treatment of cholera or severe haemorrhage, or as a vehicle for salvarsan or anaesthetics has fully proved their value. It appears, however, to be widely believed that the intravenous or subcutaneous injection of large quantities of saline is an entirely innocent procedure. On looking for trustworthy evidence in support of this belief, one is met with two striking facts: (1) The great variation in the quantities of liquid injected, together with an absence of any serious attempt to graduate the volume of injection according to the body weight; (2) the wide variation in the absolute quantities of salt injected.

For some considerable time we have been studying the effects on animals of injection of saline solutions of various strengths, of distilled water, and of other liquids. Though our experiments were undertaken for a very different purpose, the results may have some value in helping us to determine how far the administration to man of saline injections can be safely carried. We have, therefore, briefly summarized those points that bear on this question. We find that the injection of large quantities of normal or slightly hypertonic saline solutions into a vein or under the skin is not nearly so innocuous a proceeding as is often supposed. The quantities injected were usually far smaller than the quantities, relatively to body weight, that are frequently given in man. The undesirable effects that we encountered include fever, rigors, sub-normal temperatures, diarrhoea, intestinal haemorrhage, Cheyne Stokes

breathing, convulsions, and sudden death. The last two we have only met with after injection of strongly hypertonic solutions, and, except in the case of young animals, only when the injection has been given rapidly. For all practical purposes of injection in man these accidents may probably be neglected, though their occurrence in animals accentuates the need for caution in human injections. The other accidents mentioned occur very frequently after injection into a vein or under the skin of normal or slightly hypertonic solutions. The grave sequel to large saline injections (in man) of pulmonary oedema has recently been noted in this JOURNAL.¹ To this list of clinical drawbacks to saline injections in animals may be added the onset of glycosuria recorded by Martin Fischer and quoted by Bingel.² We must also note the histological changes observed by other workers on this subject. Raum,³ for example, showed that intravenous injection of the dog with normal saline was followed by vacuolation of the liver cells. Allbrecht⁴ found that the red cells of animals were damaged by large injections of normal saline; Rössle⁵ observed degenerative changes in the heart muscle and capillary walls of animals similarly injected, and Hössli⁶ found that saline injections caused in animals fatty changes in the heart muscle and kidneys. We cannot here discuss in detail all of these effects of saline injections, but one result—namely, fever—we may refer to, because it illustrates the points on which we wish to lay stress. It has long been known that injections of saline into a vein or under the skin in man or animals may set up fever. In the last two years several papers have appeared on this subject in Germany, and in them it is often assumed that the fever which follows injection of saline is solely due to the salt injected and not to the water in which the salt is dissolved. In consequence, therefore, of the absence of proper controls, no constant attention has been paid to any relation between the extent of the fever produced by injection and the ratio of the volume of water injected to the weight of the animal receiving the injection. It is, therefore, frequently stated that the fever is proportionate in extent to the amount of salt injected. We find, however, that this is not the case, and that if certain precautions, which we shall later refer to, are taken with regard to water used in making up the solutions no fever whatever follows the injection of saline solutions. We find, on the other hand, that consideration of the ratio of the volume of liquid injected containing the salt to the body weight is of the greatest importance. In other words, we are able to show that some of the bad effects produced by saline injections are more easily brought about by injections of

distilled water, as one of us has already shown.⁷ Whether any benefit ensuing from saline injections is due to the salt, or to the water, or to both, it is certain from our experiments that in animals injected with sufficiently large volumes of saline, the influence for evil of the water injected cannot be ignored. The importance, therefore, of ensuring that the total volume injected does not exceed a certain fraction of the body weight is manifest. The basis of this statement is the fact that in rabbits injection of volumes of saline below what we may call the critical point of safety produces no apparent ill effects, whilst the injection of quantities above this point does produce undesirable effects, and that in a very short time. What we have so far said relates mainly to single injections. When multiple injections are given, and still more when continuous injection is practised, unpleasant results ensue in proportionate degree. In attributing, however, the fever in our control experiments only to the distilled water injected, there is a possibility of error.

In a recent paper by Wechselsmann,⁸ attention is called to the dangers of intravenous injection of saline solutions of salvarsan. This observer believes that many of the unpleasant effects which he found to follow the injection of salvarsan were directly due to bacteria he found in the distilled water of the solutions he injected. He states that the rigors, fever, diarrhoea, vomiting, cyanosis, and headache which so many have reported are due to the fact that the water used is not sterile, and that they can be avoided by filtration of the water through hard filters and by heating in an autoclave. He bases his belief on the fact that the water he used when plated out gave a luxuriant growth of a Gram-staining spore-bearing bacillus. He admits, however, that slight fever still occurred in 19 per cent. of his cases even after filtration and heating of the water. If Wechselsmann's finding of living bacteria in the water that he supposed to be sterile applies to all distilled water prepared and stored with the ordinary precautions observed in all laboratories, it is clearly a discovery of the greatest importance. It immediately concerns, in fact, every clinical or laboratory worker who believes that distilled water, collected and protected from infection with all the care incident to a surgical operation, is innocent of bacterial protein. Its interest to those who give injections of salvarsan in saline is evident. It equally affects all who inject solutions of saline in order to prevent or combat operative shock, or to those who treat cholera or other diseases by the same method, as well as to those who administer anaesthetics by the intravenous route. It also seriously affects those

engaged in studying experimental fever by the injection of solutions in which water supposed to be protein free is the solvent. And it is difficult to believe that it does not also affect the interpretation of serum reactions in the test tube, or in the living animal if the serums are diluted with water or saline. Finally, it cannot but affect the interpretation of the results of using leucocytes or vaccines for purposes of diagnosis or treatment after repeated washing with solutions containing unsuspected organisms.

So far-reaching, in fact, are the possibilities involved by Wechselmann's observations that it becomes very necessary to determine if distilled water collected and kept under ordinary conditions of surgical cleanliness is or is not sterile. We have therefore gone very fully into this matter. Detailed observations will be found at the end of this paper. We find that if ordinary precautions are taken to prevent infection the number of organisms that can be cultivated from the distilled water we examined is quite insufficient to account for the fever. The possibility of multiplication of non-spore-bearing organisms in the body after injection is excluded by the custom of boiling all solutions of saline before injection, and the absence of spore-bearing organisms in the samples of water examined was shown by unsuccessful cultivation. We were also able to exclude the possibility of fever being caused by unaltered protein of living organisms, because the number of bacteria found on cultivation was in all cases too small. Control observations with virulent typhoid organisms showed that much larger numbers must be injected of these to produce fever. We found, moreover, that filtration or centrifuging was ineffective in removing the fever-producing properties from any of the samples of water allowed to stand that we examined. On the other hand, we find that if water distilled in a suitable glass retort is at once injected into animals it does not produce fever. On the contrary, freshly distilled water tends to produce on injection a marked fall of temperature. We are forced, therefore, to the conclusion that the results reported by Wechselmann must have been due to gross contamination of the water he employed. How, then, are we to explain our own results? We are driven to the suggestion that the fever which always follows the injection of suitable quantities of distilled water allowed to stand is caused by soluble products, perhaps of bacterial origin, and is not directly due to bacteria as such or to unaltered protein. From our innumerable experiments with distilled water injections, which will be published in due course, we believe that Wechselmann's inability to prevent fever in 19 per cent. of his cases

proves that he has not been able to get rid of the fever-producing properties that we find in all distilled water allowed to stand. In salvarsan therapy it must be remembered that the quantities of saline injected are relatively small. If, therefore, he had studied the ratio of volume of injection to body weight he would have found, as we have done, that the percentage of cases giving a febrile reaction after larger injections would, in spite of filtration and heating, have been considerably greater. To conclude, we think—

1. That saline injections as at present administered are by no means free from risk, especially when they are large.

2. That the cause of the toxicity of distilled water that is allowed to stand in sealed sterile vessels is not yet explained.

3. That neither centrifuging, filtration through cotton-wool or bacterial filters, nor boiling is sufficient to prevent the fever that follows saline injections.

4. That distillation in a sterile Jena retort of all water used in the preparation of saline solutions, followed by immediate injection, is at present the only reliable method of ensuring that no fever follows the injections.

It has been proposed that instead of injecting normal or slightly hypertonic solutions of saline, Ringer's solution may be a good substitute, on account of the inhibitory effect on fever that it is supposed to possess. Before, however, this can be accepted, fresh experiment must be undertaken with this solution, made up with water distilled in the manner suggested, and tested by injection for a toxicity.

EXPERIMENTAL DATA.

1. Water obtained by distillation from a Jena glass retort, if injected at once, produced no fever. The amounts of water injected varied from one-five-thousandth to one-twenty-fifth of the body weight. This water, after standing for five hours, on plating gave four organisms per cubic centimetre. The total solids, kindly estimated by Dr. Young from 225.0 c.cm., were 1.7 mg.

2. A sample of the above water was autoclaved in a plugged Jena flask for ten minutes at 120°C., and was allowed to stand at room temperature for forty-eight hours. On injection it produced marked fever. It was boiled before injection twice.

3. Distilled water as used for routine purposes was collected from a copper still into a closed tin-lined copper receiver, and immediately

injected after boiling. It produced slight but constant rises in temperature. This water contained 160 organisms per cubic centimetre. The total solids from 225 c.cm. were 2 mg.

4. Water from the same source allowed to stand for a few days or weeks in clean stoppered bottles constantly produced high fever after boiling. The amount of fever was always nearly proportionate to the volume injected. Several hundred observations were made. The total residue from 225 c.cm. of water that had stood for many weeks was 2.2 mg. Two different samples were plated out. (a) The water that had stood for two months gave 40 organisms per c.cm. (b) The water that stood for ten days gave 73,000 organisms per c.cm. The counts in all cases were made from agar plates grown for forty-eight hours at 37° C. Counts made from agar plates at 22° C. were taken on two occasions and gave very similar results. The water that contained 40 organisms per c.cm. gave a greater fever than that which gave 73,000 organisms. Both these waters were centrifugalized for thirty minutes in Dr. Martin's turbine centrifuge. Small quantities of the upper layers immediately below the surface invariably gave rise to high fever. The invisible deposit when injected in 0.50 c.cm. of water gave rise to no disturbance of temperature.

5. Distilled water that had been allowed to stand gave rise to fever after filtration either through a Doulton filter or through cotton-wool. Two filters were used. One was new, the other had been used several times. In both cases after filtration the water showed on analysis considerable quantities of calcium. Before filtration the ammonium oxalate test gave no reaction. The same results were obtained with a Berkefeld filter.

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The Relation of Salvarsan Fever to other Forms of Injection Fever.

By EDWARD C. HORT and W. J. PENFOLD.

WHEN injections of salvarsan in saline began to be widely employed, it was soon found that fever might ensue. Although many theories have been advanced to account for this accident none of them will explain all the facts. On the contrary, we find that salvarsan fever is so closely allied to other forms of injection fever that we thought that this relationship might afford an interesting subject for discussion. In order, however, to make clear the points we wish to make a slight historical digression is necessary.

In 1906 it was shown by Kottmann [4] that injection of saline into man might give rise to fever, and this was confirmed by Schaps [6] in 1907. Since then many workers have shown that injection of animals with saline may have the same effect. Salt fever, therefore, as it was called, became established in the literature, and numerous theories sprang up as to its meaning, all based on the belief that salt was the active pyrogenetic agent. Under this heading also really belongs the fever that may follow the injection of saline in intravenous anaesthesia, surgical shock, hæmorrhage, cholera, and so forth, though often the condition demanding treatment has been held responsible for the fever. Fever after injection of sea-water is a recent addition to this group. In 1910 one of us found [1] that the injection of animals with sterile water also caused fever, which therefore came to be known as water fever. In the belief that the water was absolutely pure this worker maintained that water fever was an auto-intoxication, and in August, 1911, suggested [2] that salt fever and salvarsan fever were in reality only different forms of water fever. In the last few years it had also been found that the injection of certain other substances, such as sugar or tissue extracts in water or saline, would give rise to fever. Accordingly carbohydrate fever, tissue fever, protein fever, ferment fever, and

so forth, all took their place in the literature as definite clinical types. In each case the substance injected was credited with specific pyrogenetic properties. And finally came salvarsan fever. Here, then, we have all these types—water fever, salt fever, sugar fever, ferment fever, tissue fever, anaesthesia fever, surgical fever, sea-water fever, and salvarsan fever, each apparently the result of different causes.

In December, 1911, we showed [3], so far as we know for the first time, that there is good reason to believe that some of these different types of fever are due to a common cause. Our experiments leading to this result were suggested by a theory advanced by Wechselsmann [7] that salvarsan fever is due to gross infection, demonstrable at the time of injection, of the solutions of salt and salvarsan. Wechselsmann's theory as to the cause of salvarsan fever was subsequently endorsed by

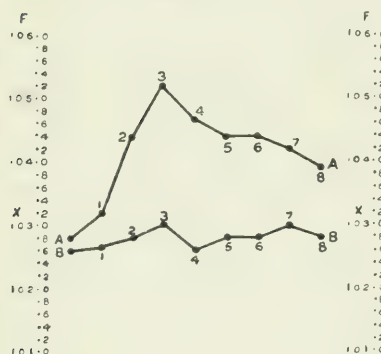


CHART 1.

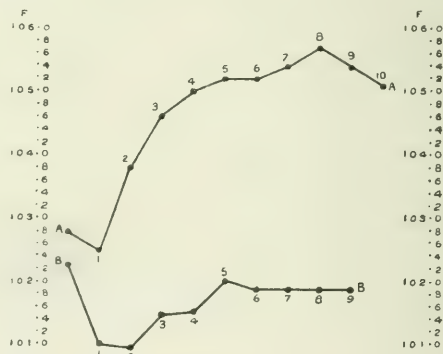


CHART 2.

Chart 1.—A, Rabbit, 2,083 gm. injected intravenously with water containing fever toxin and from which 73,000 organisms per cubic centimetre have been removed by centrifuge. Injection ratio 1 in 211. B, Rabbit, 2,983 gm. injected with deposit in pure water of 6,000,000 organisms thrown down from above water after thirty minutes in centrifuge tubes. Quantity of water injected 0.50 c.c. Observations every 30 minutes.

Chart 2.—A, Rabbit, 2,500 gm. injected intravenously with *c.* 40 million heated *Bacillus typhosus* organisms. B, Rabbit, 2,448 gm. injected intravenously with *c.* 50 million saline-grown organisms after heating and centrifuging from saline injection of which in ratio 1 in 50 produced no fever. Interval between observations 30 minutes.

McIntosh, Fildes, and Dearden, working in Dr. Bulloch's laboratory at the London Hospital, as a result of independent observation. The theory at first sight seemed to afford a clear explanation of salvarsan fever, because the liquids examined, either before or after the addition of salt, were reported to contain large numbers of organisms. Moreover, if saline made with freshly distilled water were used, it was found that

salvarsan fever did not occur. This was in one instance [5] also true after injection of a filtered sample of sterilized saline made with water that contained before filtration large numbers of organisms, and the apparent effect of the filter in preventing fever was therefore used as an argument that salvarsan fever and salt fever are necessarily due to the actual presence of micro-organisms. No control experiments, however, were cited by these observers to show that organisms grown in pure saline are, if injected immediately after heating, in actual fact themselves capable of producing fever. And from the evidence adduced [5] in support of the statement that filtration will render toxic saline atoxic, it is not clear whether the toxicity, in terms of fever, of the unfiltered sample of water with which the saline was made up had or had not been demonstrated. The theory, in fact, appeared to be based on the

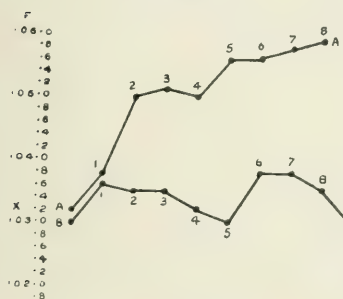


CHART 3.

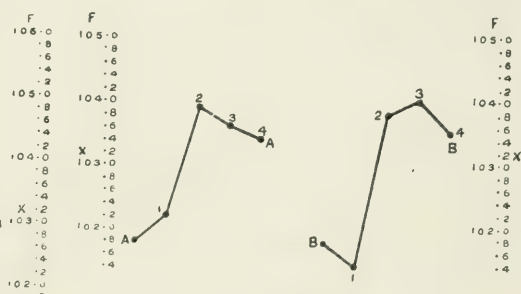


CHART 4.

Chart 3.—A, Rabbit, 2,324 gm. injected intravenously with 40.0 c.c. water containing filtrable fever toxin and 40 organisms per cubic centimetre. Injection ratio 1 in 58. B, Rabbit, 2,448 gm. injected intravenously with 49.0 c.c. saline containing 950,000 organisms per cubic centimetre, but no filtrable fever toxin. Injection ratio 1 in 50. Both liquids injected immediately after heating. Interval between observations 30 minutes.

Chart 4.—A, Rabbit, 2,278 gm. injected intravenously with 10.80 c.c. water containing 40 organisms per cubic centimetre and filtrable fever toxin. Injection ratio 1 in 211, before filtration. B, Rabbit, 2,335 gm. injected intravenously with 11.0 c.c. from the same water. Injection ratio 1 in 211, after filtration. Interval between observations 30 minutes.

assumption that the presence of a large number of dead organisms is in itself sufficient to cause fever, whatever their source.

We therefore conducted control experiments to see if unbroken bacterial protein is necessarily pyrogenetic, and if the ordinary bacterial filter does actually render toxic solutions atoxic. We centrifuged 225 c.c. of heated water, and 54 c.c. of heated saline, containing respectively 73,000 and 950,000 organisms per cubic centimetre, and injected the deposits. In neither case (Charts 1 and 2) did fever result. We also

injected 50 c.c. of a specimen of saline containing nearly 50 million organisms (Chart 3). This saline was autoclaved immediately before injection. Again no fever resulted. Finally, we injected samples of water and of saline, both grossly infected, before and after filtration through white Doulton candles, and found that their fever-producing properties were practically unimpaired by passage through the filter (Chart 4).

Clearly, therefore, the theory that salvarsan fever is necessarily due to the actual presence in the solutions of dead micro-organisms capable of removal by hardware filters is not supported by our experiments. We do not, of course, assert that what we found to be true of organisms grown in water or saline in our laboratory is neces-

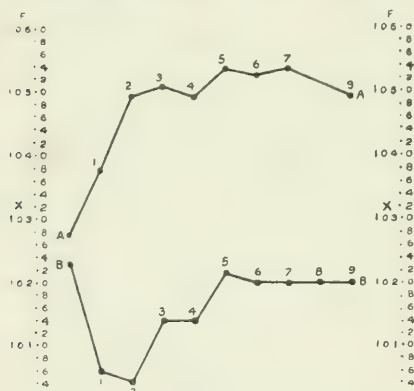


CHART 5.

Chart 5.—**A**, Rabbit, 2,324 grm. injected intravenously with c. 42.0 c.c. water containing filtrable fever toxin and 40 dead water-grown organisms per cubic centimetre. Injection ratio 1 in 50. **B**, Rabbit, 2,448 grm. injected intravenously with 49 c.c. pure saline containing no filtrable fever toxin but c. 1 million dead saline-grown organisms per cubic centimetre. Injection ratio 1 in 50. Interval between observations 30 minutes.

Chart 6.—Distilled water, 1 in 5,180; 2,526 grm., **A**, 0.48 c.c. water.

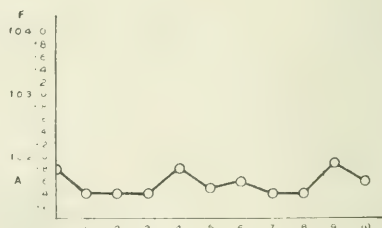


CHART 6.

sarily true of all organisms grown in water or saline. Injection of heated organisms that have been grown on nutrient media is known to give rise to fever, and from this fact no doubt arose the assumption that injection of organisms grown on water or saline and then heated must necessarily have the same effect. Since, then, this theory of salvarsan fever appears incomplete, explanation of its cause must be looked for elsewhere.

In December last [3] we showed that the pyrogenetic function of ordinary distilled water is often inversely proportional to the number

of organisms present. Water, for example, containing forty dead organisms per cubic centimetre produced high fever, whilst an equivalent quantity of saline containing nearly a million dead organisms per cubic centimetre produced none (Chart 5). We also pointed out that an important factor in the production of fever after injection of sterilized water or saline is the presence of a heat-stable substance, of unknown source, incapable of removal by the centrifuge or the ordinary bacterial filter (Charts 6—11).¹ We now find that this pyrogenetic body is to a great extent, though not entirely, held up by Martin's gelatine filter. It is therefore a colloidal substance of small molecule. Since we could demonstrate its presence both in liquids grossly infected and in liquids practically bacteria-free, the problem is to explain how the contamination arises. We find that if water be collected from the ordinary distilling apparatus found in laboratories and pharmacies and

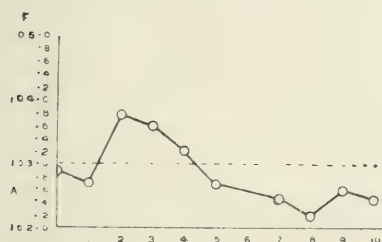


CHART 7.

Chart 7.—Distilled water 1 in 2,000 ; 2,250 grm., A, 1.10 c.c. water.

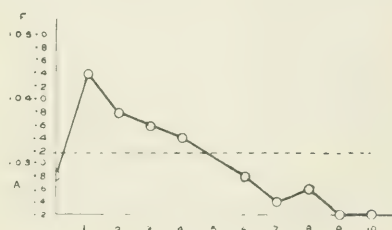


CHART 8.

Chart 8.—Distilled water 1 in 1,375 ; 2,363 grm., A, 1.71 c.c. water.

at once injected after sterilization, marked fever may follow, in spite of the fact that the water is sterile. This also applies to saline made from this water. We also find that water freshly distilled from a glass retort, and at once injected, does not contain this filter-passing pyrogen, and therefore does not cause fever, whether salt and salvarsan be added or not.

One possible suggestion, therefore, as to the explanation of salvarsan fever is as follows: The receiving tank in a good closed distilling apparatus is impervious to air infection except through the joint with the condenser, which being generally of cork should prevent entry of organisms in the absence of reverse currents. When the apparatus is

¹ Charts 6—10 show the progressive amounts of fever produced by increasing quantities of toxic sterile water when injected in strict relation to body-weight, Chart 6 showing no fever owing to the small quantity injected.

in use there is, of course, no reverse current. As soon, however, as distillation for the day is ended cooling takes place, and a reverse current, the action of which can be watched, is set up. Water in the receiving tank in consequence becomes infected from the air by aspiration. The next time the apparatus is used, unless the tank be meanwhile emptied, the freshly distilled water coming from the condenser is liable to contamination by passage through a residue that is readily shown to be pyrogenetic. Very few organisms can be found in the latter, because if the still is in daily use they are destroyed by the high temperature of the water coming from the worm when the condenser has become hot. The net result is that through repeated small increments of infection, when the apparatus is cooling, the water in the receiving tank becomes charged with this heat-stable toxin. This substance appears, in fact, to be a degradation product of air-borne

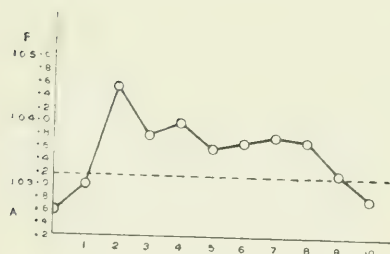


CHART 9.

Chart 9.—Effects of distilled water, 1 in 490; 2,450 grm.; A, 5.0 c.c. water injected.

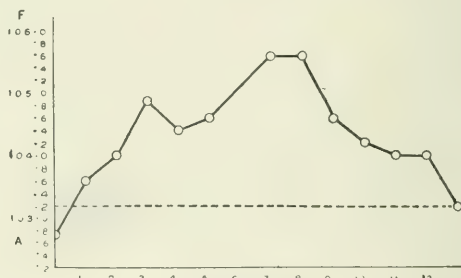


CHART 10.

Chart 10.—Distilled water, 1 in 58; weight of rabbit, 2,926 grm.; A, 50.40 c.c. water.

organisms, themselves no longer to be found. Subsequent infection of this water in the laboratory, or of saline made from it, may of course occur if good conditions are not observed. Unless, therefore, control injections be made before the onset of secondary infection, a totally erroneous interpretation may be put on the fever that may follow injection of saline made with this water after secondary infection has occurred. If, on the other hand, water freshly distilled from glass, and proved to be free from this pyrogenetic substance by injection, be mixed with salt and exposed to laboratory air for several days, and injected immediately after autoclaving, fever may or may not result. If, however, a sample of this saline, if infected, be autoclaved, and after an interval of days be then injected, fever follows, apparently because in the interval degradation products have now been extracted. The con-

clusion, therefore, appears to be that protein extractives derived from air-borne organisms are not present in sufficient quantity to cause fever if a sufficient time has not been allowed to elapse to permit of extraction. If, on the other hand, the organisms are killed, and if adequate time be allowed for extraction, degradation products make their appearance and fever will then follow injection (Chart 12).

Whether this explanation prove eventually to account for all the facts or not, it is certain that demonstration of the sterility of any given sample of water or saline affords no guarantee of the absence of this heat-stable pyrogenetic body. The presence or absence of this substance can be readily shown in an hour's time by the injection of rabbits, but unless the volume of injection be graduated according to body-weight no reliable inference can be drawn, as our charts show.



CHART 11.

Chart 11.—A rough curve illustrating the points shown in Charts 6—10.



CHART 12.

Chart 12.—**A**, Rabbit, 1,970 gm., injected intravenously with 78.0 c.c. of water. **B**, Rabbit, 2,083 gm., injected intravenously with 85.0 c.c. of water. **C**, Rabbit, 2,112 gm., injected intravenously with 87.0 c.c. of water. Injection ratios in all three, 1 in 25. Interval between observations 30 minutes. In **A** the water was taken direct from the receiving tank. In **B** the water was collected direct from the worm. In **C** the water was collected direct from a glass retort without the intervention of worm or receiving tank.

It is, we believe, this substance, whatever its origin, that is largely responsible for salvarsan fever, salt fever, water fever, and many of the other types of injection fever referred to. This belief is based partly on the work we have outlined. It is also based on numerous experiments by which we find that it is very difficult to produce injection fever of any kind, apart from bacterial fever, if the water and saline used as solvent or vehicle do not contain this pyrogenetic substance. These

experiments, however, we are not at liberty to refer to further here as they have been submitted to another Society. The importance of recognizing its existence is, however, obvious. Although the explanation we have offered of its appearance in water and saline seems to be supported by the facts we have quoted, we cannot as yet be absolutely certain that this filter-passing toxin is of bacterial origin.

The method of preventing contamination of water or of saline by this fever toxin is fortunately simple. The use of freshly distilled water that has been collected from a receiving tank in connexion with the ordinary metal still is, as we have seen, not permissible, unless the tank is emptied and cleaned before use. If water be used immediately after distillation from a glass retort, fever does not follow the injection of salvarsan in saline made from it. If storage be desired, water distilled from glass should be at once collected direct into sterile vessels, which should then be immediately hermetically sealed and autoclaved. Samples of water prepared in this way on examination several weeks afterwards did not produce fever, either alone or in combination with salt. The use of sealed ampoules containing sea-water or saline has been practised in France for some years. In the case of saline, and often in the case of sea-water, each ampoule is labelled to the effect that it has been autoclaved. We recently examined some of these, and found that many of the ampoules contained saline and sea-water that were pyrogenetic. Either, therefore, the water when inserted already contained this pyrogenetic substance, or delay occurred between distillation and autoclaving. If delay does occur, or if the water be taken from an infected still, autoclaving is a distinct disadvantage, because the extraction of toxic degradation products appears to be then facilitated.

In discussing the causes of salvarsan fever it has often been suggested that a pyrogenetic substance may be set free from the spirochætes destroyed, and that salvarsan fever may be, wholly or in part, a spirochæte fever. Before the latter view can be accepted it will be necessary to inject a suspension or extract of washed spirochætes in pure saline or water. Until, however, this be done it is not possible to explain salvarsan fever by contamination of the water or saline alone, as Dr. McIntosh and his colleagues have pointed out. The evidence they have so far brought forward of spirochæte fever, though highly suggestive, is not convincing, because the two cases they quote were children. It is therefore possible that the fever observed in these two cases after injection of salvarsan was due to slight toxicity of the solutions which was not detected by injection of adults. In other

words, as we have shown, it is not safe to assume that any given saline is atoxic because it contains no organisms, and produces no fever when injected into a man weighing, say, 14 st. Injection of the same volume of the same saline into a child weighing, say 6 st., may, if the water has been taken from the ordinary closed still, contain the pyrogenetic substance. Constant volume and inconstant weight are a fruitful source of error. On the question of spirochæte fever it is perhaps worth notice that Wechselmann met with no fever after injecting 150 cases with salvarsan in pure saline, a fact which throws some doubt on the ability of spirochætes to produce fever.

We conclude, therefore: (1) That there is at present no evidence that salvarsan fever is necessarily due to injection of organisms grown on water or saline; (2) that the presence in water or saline of a filter-passing pyrogenetic substance, which may or may not be a product of bacterial protein, is an important factor in the production of salvarsan fever and of many other types of injection fever.

In saying this, however, we wish to make it clear that our ability to demonstrate the fever-producing properties of this unsuspected contamination of watery solutions is the direct outcome of the observations made by Wechselmann and his supporters. Speaking for myself alone, if I had been aware of its existence when I advanced the autolytic theory of fever, I should not now be in the unenviable position of demonstrating to be false what I then believed to be true. To save other workers from a similar fate I am bound, therefore, to disclose the fallacies involved.

Note.—In speaking of fever throughout this paper we refer only to the presence or absence of fever occurring within one to six hours of injection.

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A Critical Study of Experimental Fever.

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(Communicated by Dr. C. J. Martin, F.R.S. Received February 17,—Read March 14, 1912.)

(From the Lister Institute of Preventive Medicine.)

The injection of solutions of salt into man and animals by the subcutaneous or intravenous route has in recent years become a common practice. In many cases the procedure is followed by fever, as was shown in man by Kottmann(1)* and by Schaps(2). If distilled water alone be injected in large quantities the same accident may happen, as shown(3) by E. Bergmann in 1868. In 1910 the injection of small quantities of water into animals was found(4) to have the same effect.

The cause, however, of what are known as "water fever" and "salt fever" has till recently(5) not been satisfactorily explained, though many theories have been advanced.

In 1911 it was suggested by Wechsellmann(6) that the fever so often met with in man after injection of sterilised saline containing salvarsan is due to gross bacterial infection of the saline, demonstrable just before sterilisation. This view was based on the discovery of numerous organisms in his unheated solutions, and on the fact that fever no longer followed injection if he dissolved his salt in freshly-distilled water. This suggestion as to the cause of salvarsan fever was also adopted by McIntosh, Fildes, and Dearnont after independent confirmation in Dr. Bulloch's laboratory of Wechsellmann's observations. The theory appeared to us to have an important bearing on the wider question of water fever, and of salt fever in general, and we therefore conducted several experiments on animals, publishing(5) our results in December, 1911.

We were able then to confirm the statement that solutions of salt are apt to exhibit pyrogenetic properties that do not belong to solutions made with freshly-distilled water. We found, however, that in the case of water these properties bore an inverse relation to the number of organisms present in the specimens we examined, and by control experiments in animals we showed that to a great extent they were primarily due to the presence in the water of a substance indestructible by prolonged heating at 120° C. We could not remove this fever-producing substance either by filtration through white

* Numerals in brackets refer to List of References at end of paper.

Doulton filters or by the use of the centrifuge, as shown by subsequent injection of the water. The actual presence therefore of organisms at the time of injection of sterilised saline did not appear to be the sole cause of salvarsan fever, nor of "salt fever" in general.

Since reporting these results we have studied the question more fully. Injection of the centrifugalised deposit from 250 c.c. of water shown to be pyrogenetic, and to contain 73,000 organisms per cubic centimetre, produced no fever. This was also true of a deposit from 75 c.c. of saline shown to be pyrogenetic, and to contain before the use of the centrifuge 950,000 organisms per cubic centimetre. In both cases the medium employed for injection of the organisms was first shown to be free from the heat-stable fever-producing body referred to. On the other hand injection of suitable quantities of water containing this body, but only 40 to 160 organisms per cubic centimetre, produced fever whether salt were subsequently added or not. This body is held back by Martin's* gelatine filter, a fact which shows that it is a colloidal substance in a fine state of dispersion. We conclude, therefore, that contamination by this body of water before mixture with salt is a more important factor in salvarsan fever than we had realised.

The unexpected presence of this hitherto unrecognised body must to some extent vitiate deductions drawn from previous work on the causation of fever which is the sequel to injection of a variety of substances dissolved or suspended in water.

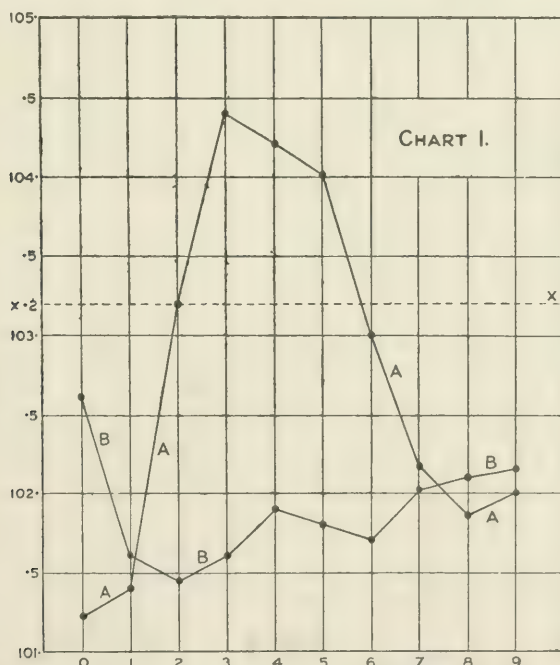
We have therefore determined to what extent the fever which had been known to follow injection of salt, sugar, fibrin ferment, and tissue extracts was due to this contamination.

Experimental Methods.

Before dealing with our evidence, it is necessary to refer to the current belief that it is impossible to study experimental fever with any approach to accuracy. The number of variants is admittedly great. Full experimental data dealing with the most important will be published in due course. They include the effect on body temperature of disease, age, sex, weight, breed, food supply, environment, exercise, rest, repeated thermometric observation, and other factors. Prolonged study has taught us that to establish broad effects, such as the ability of any given substance to produce fever on injection, knowledge of all the variants is necessary but sufficient. On the other hand, no comparative observations can be relied on unless the ratio of the volume of an injection (including weight of material dissolved or suspended in injection fluid) to body weight is kept constant. In every

* C. J. Martin, "Gelatine Filter," 'Journ. Physiol.,' 1896, vol. 20.

experiment already reported by one of us this injection ratio is stated. In all experiments here cited the same rule is observed. In our experiments we found it necessary to select animals as far as possible of the same age and weight, and to observe constant conditions of food supply and external temperature. All thermometric observations were taken in the rectum every 30 minutes, instruments of tested accuracy being always employed. Every observation has been taken by us, and never entrusted to assistants. The upper normal limit of the daily range of temperature in 150 healthy rabbits was found to be 103.2° F. All animals with a temperature more than slightly above this point were rejected, as well as those showing any apparent departure from health in other ways. Animals less than 1500 gm., or more than 3000 gm., in weight are unsuitable for experiment if the rabbit be used, the former weight indicating too unstable a temperature, the latter too resistant. Reference is here made only to early fever occurring within five hours of injection, and statements as to the



A. Rabbit, 2448 gm., injected intravenously with 11.50 c.c. water containing F.P.B. Injection ratio, 1/211.

B. Rabbit, 2478 gm., injected intravenously with 11.80 c.c. water containing no F.P.B. Injection ratio, 1/211.

(Interval between observations, 30 minutes.)

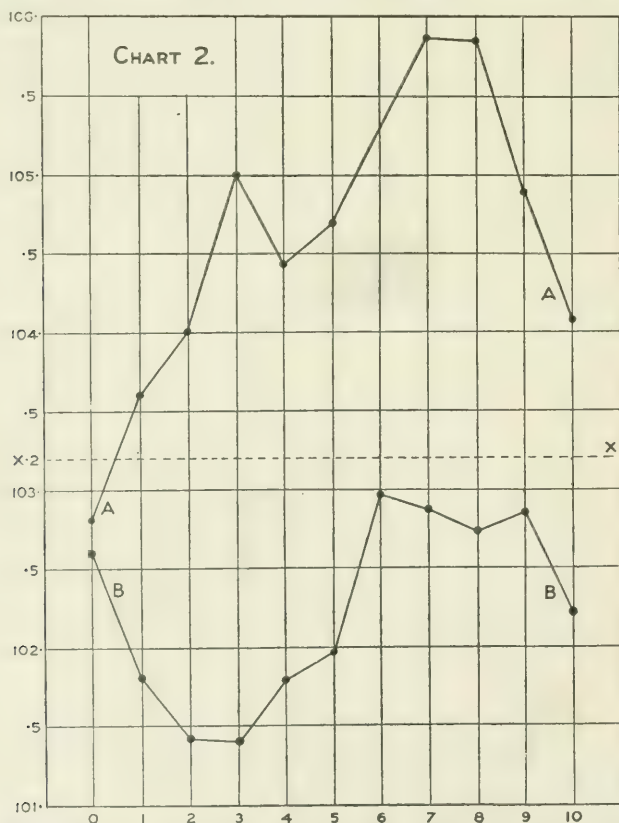
X corresponds to upper normal limit of range of temperature in healthy rabbits. This letter occurs throughout the charts.

presence or absence of fever in any given case are only made in this sense. Unless otherwise stated, all injections were made into the marginal veins of the ear.

The points we wish to establish are illustrated by charts. For the sake of brevity, we designate the fever-producing body under discussion by the letters F.P.B.

Water Fever.

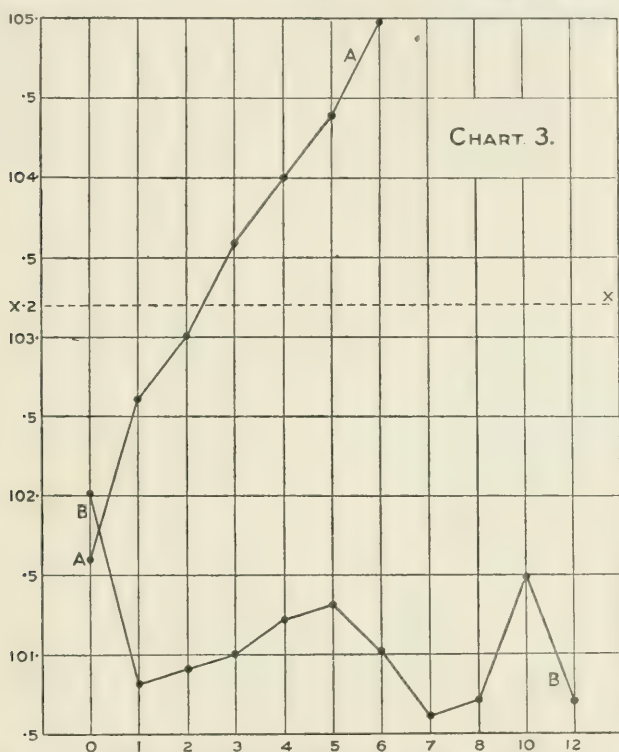
Charts 1, 2, 3 are typical instances of experiments showing the fever-producing capacity of ordinary distilled water, and its absence in the case of water freshly distilled from a glass retort and injected at once. The essential features of these charts occurred in every one of several hundred experiments in which different volumes per kilogramme were injected. Special stress is laid not only on the absence of fever in the control animals, but also on the



A. Rabbit, 2926 gm., injected intravenously with 50.40 c.c. water containing F.P.B.
Injection ratio, 1/58.

B. Rabbit, 2500 gm., injected intravenously with 50 c.c. water containing no F.P.B.
(Interval between observations, 30 minutes.)

marked fall of temperature. The different extent of fever excited by different injection ratios when one sample of water containing F.P.B. is used, is clearly seen in nearly all the charts here shown. In 1910 one of us (4) advanced the theory that water fever is largely an auto-intoxication



A. Rabbit, 2083 grm., injected subcutaneously with water containing F.P.B. Injection ratio, 1/40.

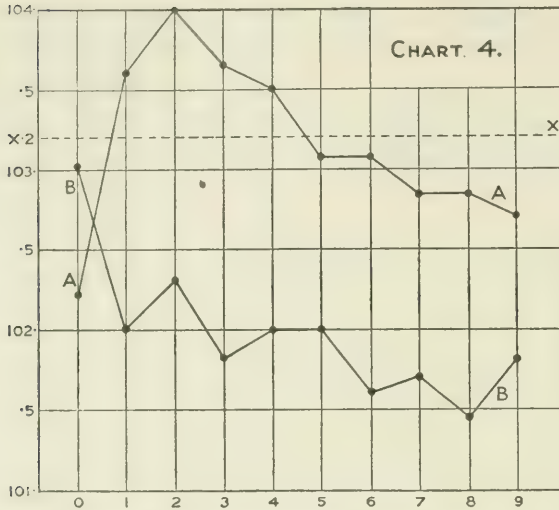
B. Rabbit, 2080 grm., injected subcutaneously with water containing no F.P.B. Injection ratio, 1/40.

(Interval between observations, 30 minutes.)

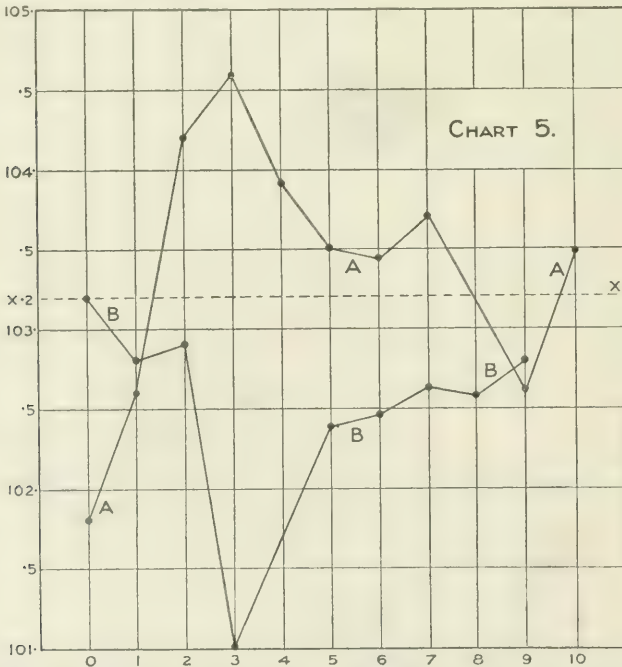
due to absorption of lytic products liberated locally by the injection of water. The evidence given in support of this view now disappears, since water supposed to be pure, and known to contain only 160 organisms per cubic centimetre, may actually contain F.P.B.

Salt Fever.

Charts 4 and 5 show that solutions of salt made with ordinary distilled water give rise on injection to fever, but that when freshly distilled water is the solvent a fall of temperature results. These effects were obtained in all our experiments up to 25-per-cent. concentrations of sodium chloride.



- A. Rabbit, 1970 gm., injected intravenously with saline made with water containing F.P.B. Injection ratio, 1/211.
 B. Rabbit, 1800 gm., injected intravenously with saline made with water containing no F.P.B. Injection ratio, 1/211.
 (Interval between observations, 30 minutes.)



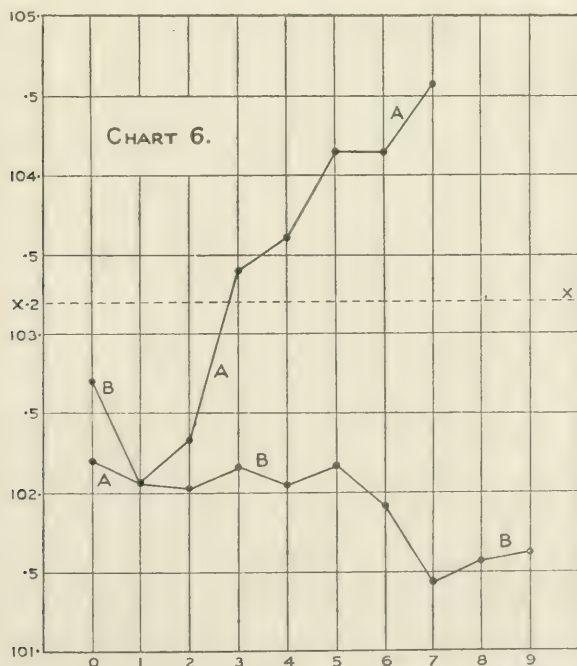
- A. Rabbit, 2551 gm., injected intravenously with saline made with water containing F.P.B. Injection ratio, 1/350.
 B. Rabbit, 2316 gm., injected intravenously with saline made with water containing no F.P.B. Injection ratio, 1/211.
 (Interval between observations, 30 minutes.)

In the experiments illustrated by the curves A the water injected was ordinary distilled water and contained 40 organisms per c.c. In the case of the experiments illustrated by curves B, the saline was made with freshly distilled water and at once injected. The results here recorded occurred in all essentials in all of the 27 experiments made. Numerous papers dealing with salt fever have appeared (7-14) in which various theories based on the view that salt was the pyrogenetic agent have been advanced. The evidence in support of these views no longer holds good.

Carbohydrate Fever (Charts 6, 7, 8).

Charts 6 and 7 show that the injection of glucose and saccharose produced no fever when the solutions injected were made in pure water.

Chart 8 shows temperatures obtained in two experiments made with lactose from different sources. The solutions were made in pure water. The upper curve shows the result with a sample of commercial lactose. The lower curve shows the result with lactose made from a catheter sample of cow's

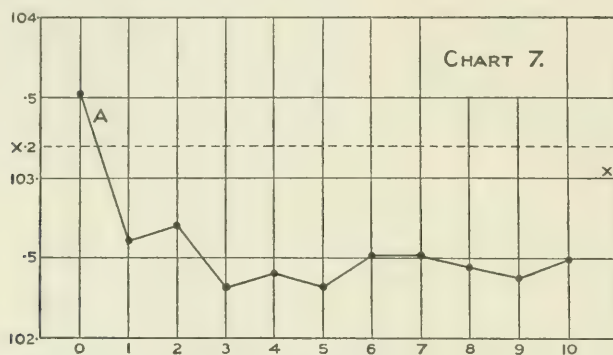


A. Rabbit, 1545 gm., injected intravenously with 8 c.c. 5-per-cent. glucose in water containing F.P.B. Injection ratio, 1/193.

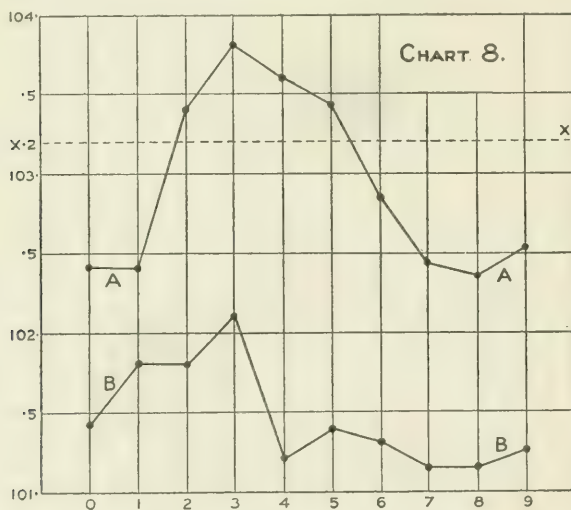
B. Rabbit, 1718 gm., injected intravenously with 8-9 c.c. 5-per-cent. glucose in freshly distilled water. Injection ratio, 1/193.

(Interval between observations, 30 minutes.)

milk. In its preparation all the reagents used were made with freshly distilled water, and the crystallisation was carried out at 0°C . For this we are indebted to Dr. McLean at the Lister Institute. With these precautions the rise of temperature was insignificant.



A. Rabbit, 1747 grm., injected intravenously with 8.8 per cent. cane sugar dissolved in 8.20 c.c. water freshly distilled from glass retort. Injection ratio, 1/211.
(Interval between observations, 30 minutes.)



A. Rabbit, 1647 grm., injected intravenously with isosmotic solution of commercial lactose in pure water. Injection ratio, 1/211.

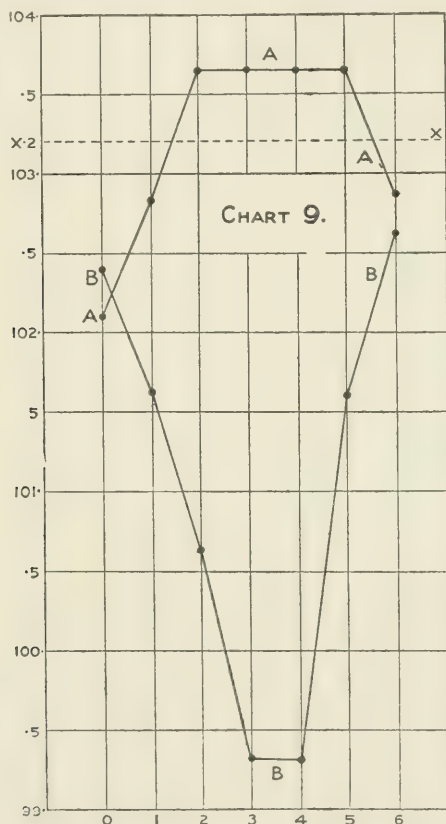
B. Rabbit, 1605 grm., injected intravenously with isosmotic solution of lactose obtained from a catheter sample of milk in the method described in text.

(Interval between observations, 30 minutes.)

The existence of carbohydrate fever (10, 15, 16), which has long been looked upon as a definite clinical type, is not supported by the above experiments.

Tissue Fever (Charts 9, 10, 11).

Chart 9 shows that the presence of F.P.B. is also responsible for the fever that follows the injection of blood laked with water, and that injection of similar quantities of blood laked with pure water produces marked fall of temperature. This fall, as will be seen, is due to the depressant effect of two distinct factors, pure water and extract of red cells.



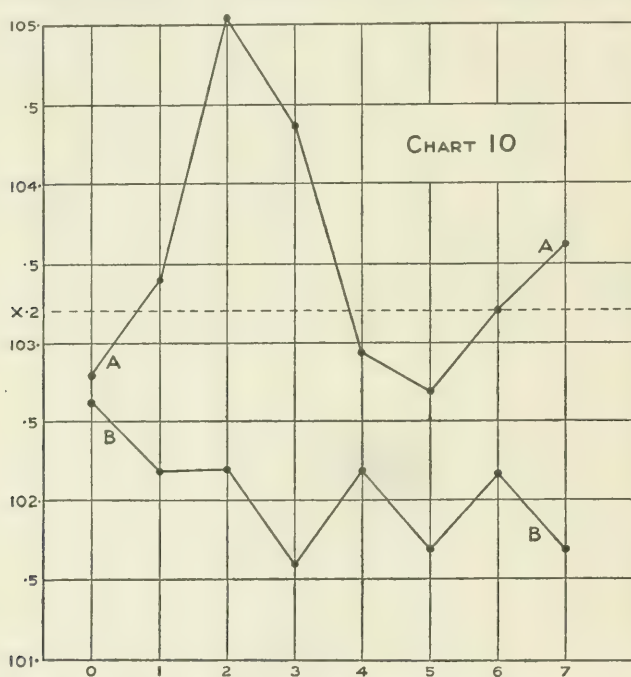
A. Rabbit, 2590 gm., injected intravenously with 4.6 c.c. rabbit blood in 15.3 c.c. water containing F.P.B. Injection ratios, 1/563 and 1/169.

B. Rabbit, 2197 gm., injected intravenously with rabbit blood in pure water. Injection ratios as in A (slightly less).

(Interval between observations, 30 minutes.)

Chart 10 shows the relative temperatures after injection of two animals with extract of red blood cells taken from another animal of the same species. The extract was made in the case of animal A by lysis of the cells in water shown to contain F.P.B., the extract for animal B being made in pure water. This chart destroys the value of the evidence of fever

due to injection of extract of red cells of the same species. The same result followed the injection of red-cell extract of another species, as well as that of red-cell extract made from the cells of the animal injected. In all cases where fever resulted the water used for the preparation of the extracts was the same and contained 40 organisms per c.c. on injection. The water employed in the preparation of the sodium citrate solutions into which the blood was shed, the saline in which the cells were washed, and for lysis of the

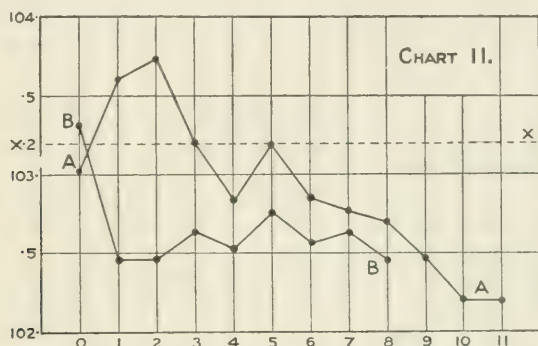


- A. Rabbit, 2785 gm., injected intravenously with red-cell extract in water containing F.P.B. Injection ratios of water and extract same as in B (figures lost).
 B. Rabbit, 2370 gm., injected intravenously with red-cell extract in water containing no F.P.B. Injection ratios of water and extract same as in A.

(For details of preparation of extract, *vide* text. Intervals between observations, 30 minutes.)

blood corpuscles was a sample containing F.P.B., and 40 organisms per c.c. The control extracts were made throughout with solutions containing pure water. The extent of fever shown is in excess of that which follows injection of a similar quantity of water containing F.P.B. This increase is perhaps due to absorption of F.P.B. during preparation of the extracts, perhaps to the action of F.P.B. on the stromata resulting in their acquisition of pyrogenetic function not previously possessed. The point requires quantitative investigation.

Chart 11 shows that the injection of fresh normal serum produces a fall of temperature, in spite of the presence of fibrin ferment. This fact gravely affects the theory of fibrin ferment fever (17-22) more especially as all the evidence hitherto accepted in support of this theory is based on injection of extracts in water that we now know to have been liable to contamination with F.P.B. Until, therefore, these experiments on ferment fever, including fibrin ferment fever, have been repeated with extracts in water proved to be free from this substance all observations on this subject require careful scrutiny.



A. Rabbit, 2211 grm., injected intravenously with 3 c.c. normal serum from another healthy rabbit. Injection 4 hours after separation. Injection ratio, 1/737.

B. Rabbit, 1932 grm., injected intravenously with 3.50 c.c. of his own serum. Injection 4 hours after separation. Injection ratio, 1/552.

(Interval between observations, 30 minutes.)

With regard to Charts 9, 10, 11 as a group it is generally believed that the injection of sterile extracts of animal tissues (23-25) may cause fever. This applies to extracts of foreign blood, of red cells, of leucocytes, or of blood serum, as well as to extracts of all foreign tissues. By the term foreign tissue is here meant tissue alien to the animal injected, even if belonging to animals of the same species. Fever is also believed to follow the injection of an animal with extracts of his own blood or blood cells, and the fever following fracture and hæmatomata have been thus explained. In all experiments, however, the extracts were prepared in liquids containing water which must now be regarded as suspect. In view of our experiments the value of much of the evidence, therefore, on which belief in this type of fever rests disappears. If reference be made to Charts 1, 2, 3 in the light of Charts 9, 10, 11 it will be seen that they afford additional evidence that the theory of tissue fever cannot be supported by lysis of cells effected by the injection of distilled water. We find this to be also true of extracts of solid organs, including the brain. The injection of fresh egg albumen also failed in our hands to give

rise to fever, a fact that has some bearing on the belief in protein fever after single injections of this class of substance in an unbroken state.

In conclusion, we submit that the existence of "water-fever," "salt-fever," "sugar-fever," "ferment-fever," and "tissue-fever" no longer rests on secure ground. That future advance in the study of fever is only possible by recognition of all the fallacies inherent to experiments involving the use of water liable to contamination with this fever-producing substance will not, we believe, be questioned.

The fact that one of us has been a victim to these fallacies is sufficient justification for the critical nature of this note.

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ON THE SURVIVAL OF SPECIFIC MICROORGANISMS
IN PUPAE AND IMAGINES OF *MUSCA DOMESTICA*
RAISED FROM EXPERIMENTALLY INFECTED LARVAE.
EXPERIMENTS WITH *B. TYPHOSUS*.

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It has recently been shown by Bacot (1911), some of whose experiments I had an opportunity of confirming (see Appendix to Bacot's paper), that bacilli (*B. pyocyaneus*) taken up by larvae of *Musca domestica* may survive the pupal stage and reappear in the imago.

In view of the importance of this fact, it appeared desirable in the interests of practical hygiene, to ascertain whether an organism such as *B. typhosus* was also able to adapt itself to the conditions prevailing in the larval and pupal interior where presumably it would have to contend with other organisms of a more hardy character.

For the purpose of the experiments detailed in this communication, ova of *Musca domestica* were kindly supplied to me by Mr Bacot.

First Series of Experiments.

Ova of *Musca domestica* were received on 5 Dec. 1910. The larvae were transferred to sterile vessels containing a layer of sterilised sand and supplied with sterilised food consisting of mashed meat, potatoes and turnips. The mash was drenched repeatedly with broth cultures of *B. typhosus*. While the larvae were still feeding, it was noted that the mash had a strongly ammoniacal smell and the question arose whether the *B. typhosus* which had been supplied so liberally to it, was the predominant organism in the mash. Examination of MacConkey plates from an emulsion of the meat showed numerous colourless colonies none of which proved to be *B. typhosus*. They all gave the

characters of an organism frequently encountered throughout this work and which I shall provisionally call *Bacillus* "A," the chief points of difference between it and *B. typhosus*, being the formation of indole and intense production of alkali in litmus milk following an initial acidity. Pure cultures of this organism gave the characteristic ammoniacal smell given by the mash in which the larvae were feeding. *B. prodigiosus* was also present in the mash.

Examination of larvae before pupation.

Four fully grown larvae were placed in absolute alcohol for 10–15 minutes and then washed repeatedly in sterile broth. Plates prepared from the mashed-up larvae gave profuse growth of colonies of *Bacillus* "A," and a few colonies of *B. prodigiosus*. *B. typhosus* was not recovered.

Examination of pupae.

On 12 Dec. twelve pupae were found. These were transferred to sterile sand in a sterile flask. The remaining larvae were washed several times in 2% lysol followed by sterile broth and finally transferred to another sterile bottle to pupate. For the bacteriological examination of pupae various methods of disinfecting the exterior were employed, similar to those mentioned in the Appendix to Mr Bacot's paper (p. 73) but it was found impossible to obtain sterile washings before the final mashing-up process. In any case, the plates prepared from the triturated pupae gave profuse growth of colonies of *Bacillus* "A." *B. typhosus* was not recovered.

The direct method of examining the bacterial content of the pupal interior was then devised (see Appendix to Mr Bacot's paper) and gave the following results in a series of pupae.

Pupae (one-day old) examined by direct method.

- No. I. Sterile.
- No. II. Profuse growth consisting of *Bacillus* "A."
- No. III. Profuse growth consisting of *Bacillus* "A" and *B. prodigiosus*.
- No. IV. Sterile.
- No. V. Sterile.
- No. VI. Only one colony on plate, viz. *Bacillus* "A."
- No. VII. Sterile.

Pupae (two days old) examined by direct method.

- No. I. Sterile.
 No. II. Few colonies, all *Bacillus* "A."
 No. III. Profuse growth of colonies of *Bacillus* "A."
 No. IV. do.
 No. V. do. + *B. prodigiosus* + *B. pyocyaneus*.
 No. VI. Mainly *B. pyocyaneus*.
 No. VII. Profuse growth of *Bacillus* "A."

In no case was *B. typhosus* recovered from the plates.

Examination of emerged Flies.

The procedure adopted in examining the adult fly was as follows: the fly after being chloroformed was immersed in absolute alcohol for 5-10 minutes and afterwards transferred to a sterile slide where the wings and legs were removed. It was then passed through the flame. The abdomen was opened with sterile needles and the intestine carefully removed and mashed up in salt solution or broth.

Results.

Fly No. I. Emerged on 19th Dec. and examined on the same day: Plates showed profuse growth of *Bacillus* "A."

Films prepared from the intestine showed enormous numbers of bacilli.

Fly No. II. (About 10 hrs. old. Had fed on sterile cane sugar and had probably evacuated its intestinal contents.) Plates proved sterile. Microscopically only a very few bacilli were observed in stained films.

Fly No. III. (24 hrs. old.) Plates sterile.

Fly No. IV. (24 hrs. old.) Plates showed *Bacillus* "A," *B. prodigiosus* and *B. pyocyaneus*.

Fly No. V. (48 hrs. old.) Pure plates of *Bacillus* "A."

Fly No. VI. (do.) (do.)

Fly No. VII. (do.) *Bacillus* "A" and *B. pyocyaneus*.

Fly No. VIII. (do.) One colony of *Bacillus* "A."

In all cases where the plates showed profuse growth of colourless colonies, a large number were picked off and carefully examined on the chance that the typhoid bacillus might be recovered, but in no case was a successful result obtained. All the colonies picked off proved invariably to consist of *Bacillus* "A."

Second Series.

Ova were received on 4th and 5th Jan. 1911 from Mr Bacot, and within 12–24 hrs. all had hatched out. On this occasion cultures were made from the egg-shells and some of the newly emerged larvae, as the negative results obtained in the first series of feeding experiments with *B. typhosus* had given rise to the suspicion that this micro-organism had only a very small chance of survival in competition with organisms contaminating the exterior of the ova and which were probably ingested at a very early period by the newly emerged larvae.

The plates showed profuse growth consisting of the following organisms, *B. proteus vulgaris*, *Bacillus* "A," *B. prodigiosus* and a *Streptococcus*. The larvae were fed with a sterile mash and kept at 25° C. until the 9th Jan. when several full-grown individuals were found.

These were washed in carbolic solution and finally transferred to a fresh sterile vessel containing sterile sand to which a liberal supply of typhoid broth cultures had been added. One, however, was not placed in the typhoid chamber but was retained for bacteriological examination. Plates prepared from it gave a growth consisting of *Bacillus* "A," *B. prodigiosus* and a lactose fermenter (*Bacillus* "B," No. 3, vide Table I). On 10th Jan., one larva was removed from the typhoid chamber and examined after careful disinfection in carbolic solution. The plates consisted mainly of *Bacillus* "A" and *B. proteus vulgaris* but one colony was found to consist of *B. typhosus*. Whether this organism actually came from the larval interior could not be decided, as the broth in which the larva had been washed after disinfection gave after incubation a growth which consisted of *Bacillus* "A," and *B. proteus vulgaris*.

Examination of Pupae.

Pupa No. I. (12 hrs. after pupation.) Plates gave pure growth of *Bacillus* "A."

Pupa No. II. (12–24 hrs. after pupation.) Profuse growth of *Bacillus* "A."

Pupa No. III. (3–4 days after pupation.) Plates consisted of *Bacillus* "A," *Bacillus* "C" (see Table), and *Bacillus* "E" (see Table).

Pupa No. IV. (4 days after pupation.) Plates consisted of *Bacillus* "A" and *B. prodigiosus*.

In no case was *B. typhosus* recovered from the pupae.

One adult fly was examined after it had fed on sterile sugar for two days.

The plates proved sterile.

Third Series.

On 24 Jan. 1911 a new batch of eggs was received and an attempt was made to sterilise the eggs before the emergence of the larvae, but before sterilisation a sample of the eggs was taken for bacteriological examination. The organisms isolated were chiefly those marked in the Table as *Bacillus* "B" No. 3, *Bacillus* "B" No. 2, and *Bacillus* "D." The remaining eggs were placed in 5% lysol for $2\frac{1}{2}$ mins., washed repeatedly in saline solution and allowed to dry as far as possible. Sterile human blood was then given them but unfortunately the eggs became entangled in the fibrin meshes and no larvae emerged.

Final Series.

On 25 Jan. another batch of newly emerged larvae was received from Mr Bacot. *These had developed from eggs which had been disinfected by a short sojourn in lysol.* The young larvae had been placed on a sterile agar slope which remained sterile. Human blood mixed with typhoid bacilli was spread on the agar and this process was repeated, the larvae being transferred to a fresh agar slope with blood every day.

Examination of larva.

On 29 Jan. one larva was removed from the slope and placed in 5% lysol for 5-10 mins. After successive baths of saline solution and absolute alcohol the larva was mashed and plated.

The plates yielded what appeared to be a pure growth of typhoid-like colonies.

Seven of these were picked off, all of which proved to be *B. typhosus*.

Films made from the mashed-up larva also showed numerous typhoid-like organisms with a few large diplococci.

Further examination of larvae.

On 31 Jan. four larvae were found dead in the condensation fluid of the agar, the excess not having been removed by an unfortunate omission. Only one larva survived. Two of the dead larvae were placed in 2% lysol for 10 hours and then plated.

B. typhosus was recovered in pure culture from each.

The surviving larva was placed on fresh sterile sand and pupated between 3rd and 4th Feb.

Examination of pupa.

On 6 Feb. the pupa was examined by the direct method. The colonies on the plates were few but all of them proved to be *B. typhosus*. They were agglutinated by an anti-serum in dilution of 1 in 2000.

SUMMARY AND CONCLUSIONS.

Although typhoid bacilli were liberally supplied to larvae of *Musca domestica*, all attempts to demonstrate *B. typhosus* in the pupae or imagines were unsuccessful, until recourse was had to disinfection of the ova. After this preliminary disinfection both larvae and pupae gave pure growths of *B. typhosus* but hitherto it has not been possible to examine the imagines.

In the experiments with unsterilised ova great difficulty was experienced in determining whether *B. typhosus* was present in MacConkey plates owing to the almost invariable occurrence of the colourless typhoid-like colonies of the *Bacillus* "A" which was evidently an organism thoroughly adapted to the conditions prevailing in the interior of the larvae, pupae and imagines.

The fact also that this organism like *B. typhosus* fermented mannite without the production of gas, rendered the search for *B. typhosus* still more difficult.

By the employment of sorbite—in place of lactose-media, a preliminary differentiation might have been possible but was not considered practicable in the first place owing to the expense of sorbite, and secondly owing to the fact that the fermentation of sorbite by *B. typhosus* though constant, is sometimes delayed. The most satisfactory plan was to pick off as many colonies as possible and inoculate them on litmus-milk. *Bacillus* "A" invariably rendered litmus-milk intensely alkaline.

From the practical point of view the main conclusion to be drawn from the experiments detailed in this communication is that the typhoid bacillus can lead only a very precarious existence in the interior of larvae or pupae which possess, at least in so far as these investigations warrant, a well-defined bacterial flora of their own.

Even under the highly artificial conditions of the final series of experiments, it was not possible to decide whether the *B. typhosus* though recoverable from the pupa was really actively multiplying in the pupal interior or gradually dying out. There was some indication that the latter was the case, as the typhoid colonies recovered from the pupa

in the one successful instance, were extremely few in number, while the larvae which had been feeding on *B. typhosus* contained enormous numbers as evidenced both by cultural and microscopical examination.

Since the above experiments were concluded, some further researches by Graham-Smith (1911) on the carriage of bacteria by flies have appeared in the form of a Local Government Board Report. This author has succeeded in recovering *B. anthracis* from blow-flies bred from larvae fed on meat infected with spores of this organism, but has failed to recover *B. typhosus* or *B. enteritidis* under similar experimental conditions.

TABLE I. *Characters of the chief organisms isolated from ova, larvae, pupae and imagines.*

(*B. typhosus* inserted for comparison with *Bacillus* "A.")

	Motility	Liquefaction of gelatine	Lactose	Glucose	Mannite	Dulcitate	Saccharose	Sorbitol	Litmus milk	Indole	Adon.	Inul.
<i>B. typhosus</i>	+	-	-	A	A	-	-	A	A	-		
<i>Bacillus</i> "A"	+	-	-	A	A	-	-	-	A - alk.	+		
<i>B. prot. vulg.</i>	+	+	-	AG	-	-	AG	-	AC pept.	+		
<i>B. prodigiosus</i> (a)	+	+	-	AG	A	-	A	A	AC pept.	-		
do. (β)	+	+	-	AG	A	-	AG	A	AC pept.	+		
<i>Bacillus</i> "B" No. 1	-	-	AG	AG	AG	AG	AG	AG	AC	-		
do. No. 2	+	-	AG	AG	AG	-	AG	AG	AC	-		
do. No. 3	-	-	AG	AG	AG	AG	AG	AG	AC	+	AG	-
<i>Bacillus</i> "C"	+	+	-	AG	AG	-	AG	-	Pept.	+		
<i>Bacillus</i> "D"	+	-	-	AG	AG	-	AG	AG	AC	-		
<i>Bacillus</i> "E"	-	-	-	A	-	-	-	-	A	-		

Notes on the organisms in Table I.

Bacillus "A." Compare with organism marked 4 B isolated by Morgan from faeces of children (see Morgan and Ledingham, *Proc. Roy. Soc. of Med. Epidem. Sect.* March, 1909, Table I). Also isolated by the writer from faeces of typhoid convalescents and examined by Morgan (see *Journ. of Hyg.* Vol. xi., No. 1, April, 1911, Table II. Organisms B 20—B 25). They are not agglutinated by typhoid or dysentery serum.

"B" No. 1—"B" No. 3. Lactose-fermenters, belonging to Groups III and IV in MacConkey's scheme (see MacConkey, "A Contribution to the Bacteriology of Milk." *Journ. of Hyg.* Vol. vi., 1906).

Bacillus "D." Compare with organisms marked No. 12 and No. 13, isolated from faeces of children by Morgan (see Morgan and Ledingham, *ibidem*).

The occurrence of *B. pyocyaneus* is explained by the fact that Mr Bacot had been experimenting with this organism. The ova had become contaminated by fly excreta containing this organism among others.

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THE ACTION OF THE COMPLEMENT-FRACTIONS ON A TROPIN-B. *TYPHOSUS* SYSTEM WITH COMPARATIVE HAEMOLYTIC EXPERIMENTS.

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¹ A brief Abstract of this communication appeared in the *Journ. of Path. and Bact.*
Vol. xvi. 1912, p. 386.

Introductory.

THIS communication contains a record of a series of experiments undertaken primarily to elucidate the action of complement and its fractions on a Tropin-*B. typhosus* system as estimated by the phagocytic index. Haemolytic experiments were also undertaken with the combined object of testing the activity of the complement-fractions and of investigating by the use of another system, certain points which arose in the course of the phagocytosis experiments. The haemolytic experiments are recorded in Part II of this communication. The term "Tropin" employed throughout the paper to designate the antibody in an inactivated immune serum which, in the absence of complement, is able to effect sensitisation of micro-organisms for phagocytosis, has been adopted solely for the sake of convenience and entirely without prejudice to the conception we have formed of its relation to the immune opsonin so-called, or to the general antigen-antibody reaction which, under certain conditions of experiment, involves the fixation of complement.

We are the more reluctant to use specific terms for anti-substances which under certain systems perform certain effects, in view of the fact that the experiences we have had in the course of this research, have impressed on us the necessity of reviewing our conception of antigen-antibody reactions in the light of the more recent work on the application of the principles of colloidal chemistry to immunity reactions. It appears probable at least that many of the inhibition and acceleration phenomena occurring in immunity reactions, will find a reasonable explanation in the laws which govern the mutual interaction of colloids to which all antigen and antibody solutions belong.

In 1907 Ferrata showed that if fresh guinea-pig serum was dialysed for 24 hours against running water, the haemolytic complement could be separated into two fractions. The one fraction was contained in the precipitated globulin, the other in the supernatant fluid. Complementing was obtained by the united action of the two fractions, but not by either fraction acting independently. Since the publication of Ferrata's paper, two other methods for the separation of the complement-fractions have been devised, that of Sachs (1909), and that of Liefmann (1909). Sachs' method consists in the partial precipitation of guinea-pig serum by the addition of very dilute hydrochloric acid. Liefmann effected a similar separation by saturation with carbon dioxide. These three

methods depend on the production of a precipitate of euglobulin. One fraction of the complement is carried down with this precipitate while the other fraction remains in the supernatant fluid. The precipitate is separated in the centrifuge, washed and dissolved or suspended in 0.85 % saline solution. Such a solution contains one of the fractions of the complement. The other fraction is contained in the supernatant fluid which is made isotonic by the addition of a suitable quantity of sodium chloride. If the separation has been successful, neither fraction has by itself any action as complement, while excellent complementing is obtained if the two fractions are mixed together.

References to later work will be found under the appropriate heading in the body of the paper.

PREPARATION OF MATERIALS EMPLOYED IN THE EXPERIMENTS.

Preparation of the Complement-Fractions.

The method used in preparing the fractions of the complement was slightly modified from that of Liefmann (1909). This method carried out in the manner which is to be described, yielded very satisfactory results.

To one volume of fresh guinea-pig serum is added nine volumes of ice-cold distilled water. The mixture is placed in a bottle of a capacity equal to about twice the volume of the contained mixture. The bottle is provided with a rubber cork which should not be too small. The bottle containing the diluted serum is placed in a can containing ice-cold water. A brisk stream of CO_2 is allowed to flow into the bottle for a few seconds. The bottle is then corked and briskly shaken. The bottle is repeatedly filled with CO_2 and shaken until the fluid is completely saturated. The mixture is then placed in the cold room or ice safe for an hour, when it is usually found that the precipitate of globulin has begun to flocculate and settle to the bottom of the bottle. The mixture is then centrifuged. The supernatant fluid is poured off and should be quite clear. The exact volume of the fluid is measured and sufficient sodium chloride is dissolved in it to obtain a concentration of 0.85 %. This represents a 1 in 10 dilution of the "end-piece" of the complement.

The deposit is twice washed in ice-cold distilled water (the deposit is suspended in ice-cold distilled water and centrifuged). The washed deposit is then dissolved in a quantity of cold 0.85 % salt

solution equal to the volume of the original 1 in 10 dilution of the serum. The result is a 1 in 10 dilution of mid-piece. The globulin deposit should dissolve readily and the resulting solution in saline should be nearly clear. It is usually necessary to filter the mid-piece solution through filter paper to remove the particles of foreign matter which get into the centrifugalised deposit. It is necessary to keep the mixture and the precipitated globulin as cold as possible during the entire process. Unless this is done, great difficulty is experienced in attempting to re-dissolve or re-suspend the globulin precipitate in saline. The only other point which requires special attention is concerned with the length of time which it is advisable to allow, after the mixture has been saturated with carbon dioxide and before it is centrifugalised. If the interval allowed be too short, it will be found that the separation has been incomplete and that the supernatant fluid still contains a demonstrable quantity of mid-piece. If too long a time is allowed, the globulin precipitate is very difficult to dissolve. After many trials it was found best to allow an interval of one hour after saturation with CO_2 and before the mixture was centrifugalised. This procedure gave in nearly every case, a satisfactory separation of mid- and end-piece.

If the supernatant fluid is allowed to stand for a few hours before the sodium chloride is added, a further deposit of globulin occurs. This readily dissolves when the salt is added. In practice the salt was always added immediately after the supernatant fluid had been separated by the centrifuge. On two occasions the supernatant fluid was intentionally kept for 24 hours without addition of salt. The second deposit of globulin was collected, washed in distilled water and re-suspended in saline. It was not found to contain any demonstrable quantity of either mid- or end-piece.

Preparation of Blood Corpuscles and Haemolytic Serum.

These were prepared in the usual manner. Sheep's blood was collected from the jugular vein and defibrinated with glass beads. The corpuscles were separated by the centrifuge and thoroughly washed.

The haemolytic serum was prepared by injecting the ear vein of a rabbit with washed sheep corpuscles. The serum obtained from the rabbit was inactivated in the usual way by heating at 56°C . for half an hour.

Immune Typhoid Serum.

This was prepared by immunisation of the rabbit with killed typhoid bacilli, the inoculation being made intravenously. Apart from the few experiments dealing with the splitting of the fresh immune serum itself by the CO₂ method, the same immune serum was used throughout. It was kept after inactivation in the cold room at a temperature of 1°–2° C.

Bacillary Emulsion.

The bacillary emulsion was prepared from an emulsion in saline of *B. typhosus* grown on ordinary agar for 18 hours at a temperature of 20° C. The advantage of using for phagocytic experiments bacilli grown at this temperature is considerable. The bacilli are of more uniform size, longer, stouter and more readily counted inside leucocytes than bacilli grown at 37° C.

PART I.

Technique of the Phagocytosis Experiments.

In a few of the earlier experiments the constituents entering into each test were mixed together directly without previous digestion of the heated immune serum with the bacillary emulsion. This technique, however, did not yield satisfactory results and recourse was had to the method of previous digestion of the immune serum + mid-piece with the bacilli—a method which has been shown to give the best results in corresponding haemolytic experiments.

The following may be taken as a sample of technique most frequently followed in the course of the experiments:

Inactivated immune serum was diluted to a concentration of, *e.g.* 1 in 500. Equal volumes of this diluted serum and of the bacillary suspension and salt solution (1 c.c. of each, *i.e.* 3 c.c. in all) were mixed together in a large test tube and placed in the incubator at 37° C. for a period varying from 30 mins. to one hour.

Similarly, equal volumes of the mid-piece solution (corresponding to fresh complement in dilution of 1 in 10), immune serum and bacillary emulsion (1 c.c. of each, *i.e.* 3 c.c. in all) were digested together for the same time. Corresponding volumes of bacillary emulsion and salt solution alone were treated similarly. At the end of the period of

sensitisation the tubes in which immune serum was present showed as a rule a greater or less degree of agglutination. Any detrimental effect of such agglutination in the subsequent phagocytosis experiments was, however, almost entirely avoided by rolling the tubes between the hands for some time when it was found that the clumps practically disappeared. Shaking was sometimes resorted to and in fact acted in the same way and more quickly but had the disadvantage of causing considerable frothing.

When all the constituents were ready the phagocytosis test proceeded as follows (in a typical experiment). By means of capillary pipettes the essential constituents were taken up one by one into capillary pipettes with rubber teats from which they were immediately blown into small test tubes and thoroughly mixed together. Immediately after mixing each test tube was placed in a to-and-fro shaker working in an incubator kept at 37° C. The period of incubation was as a rule 10, 12, 14, minutes and most frequently 12 minutes. No intra- or extracellular lysis occurred with this short period of incubation and with the materials employed. The following indicates the constituents of a typical experiment:

Previous sensitisation. "A" immune serum (1 in 500) 1 c.c. + bacillary emulsion 1 c.c. + 0.85 % saline 1 c.c.

"B" immune serum (1 in 500) 1 c.c. + bacillary emulsion 1 c.c. + mid-piece solution (corresponding to 1 in 10 fresh serum) 1 c.c.

"C" bacillary emulsion 1 c.c. + salt solution 2 c.c.

Phagocytosis test.

(1)	3 vols. salt solution	+ 2 vols. Leucocytes	+ 1 vol. "A."
(2)	3 vols. " "	+ 2 vols. " "	+ 1 vol. "B."
(3)	3 vols. end-piece (1 in 10)	+ 2 vols. " "	+ 1 vol. "A."
(4)	3 vols. " (")	+ 2 vols. " "	+ 1 vol. "B."
(5)	3 vols. complement (1 in 10)	+ 2 vols. " "	+ 1 vol. "A."
(6)	3 vols. " (")	+ 2 vols. " "	+ 1 vol. "C."
(7)	3 vols. end-piece	+ 2 vols. " "	+ 1 vol. "C."

In the tables only the essential constituents whose action is being investigated, are inserted.

It will be seen, therefore, that the fresh diluted complement or the end-piece came into action with the tropin and bacilli, or bacilli only, during the incubation stage of 10-12 minutes.

At the end of the period of incubation a small drop from each tube was taken up in a capillary pipette and placed on a slide from which films were made. The films were fixed in methyl alcohol and stained with Giemsa solution.

The number of cells counted varied from 100-300, no constant number being counted for the obvious reason that in films which presented numerous leucocytes containing no included bacilli a very much larger number had to be counted in comparison with films in which practically every leucocyte had been active.

It may be well to indicate here that T (1 in 500) or tropin (1 in 200) denotes the dilution of immune serum employed for sensitisation purposes. In the final phagocytosis experiment the concentration of immune serum in the total mixture would be (when six volumes in all were employed) 1 in 9000. Similarly the concentration of mid-piece in the total mixture would be (supposing the pure mid-piece (= 1 in 10) were digested in equal volumes with immune serum and bacilli originally) 1 in 18, or = 1 in 180 of pure original serum. Similarly where three volumes of end-piece were employed, as proved most satisfactory, the concentration in the total mixture of six volumes would be 1 in 2 (= 1 in 20 of original guinea-pig serum) and so on.

ANALYSIS OF THE EXPERIMENTAL RESULTS.

Phagocytosis Experiments.

For the following reason we have decided to tabulate the great majority of the experimental results in chronological order.

At the commencement of the work it was anticipated from experience with haemolytic systems, that the demonstration of the complementing effect of a mid-piece-end-piece complex on a Tropin-*B. typhosus* system would be a comparatively easy task. This did not, however, prove to be the case and although it would have been a simple matter to choose from the mass of experiments only those which showed these typical and more or less expected effects, we felt assured that, behind what appeared at the time to be anomalous or inconsistent results, there lay the basis of problems which further experimentation would, and did, go far to elucidate. Moreover, by investigation of such problems as they arose in the course of the phagocytosis experiments, by means of a haemolytic system (Part II), we were enabled to throw a further light on the mechanism at work in producing the results in question. There can be little doubt, however, that it will only be possible to obtain a clear understanding of the various effects, when they are considered in the light of the chemistry of colloidal solutions where very similar inhibition phenomena are met with. The difficulties we

have encountered in the course of the work, mainly from persistent inhibition effects, have opened up in fact a most interesting field for further research and we hope that the complete record of the experiments made will form a guide for other workers in this field.

Explanation of Abbreviations used in Tables.

T = Tropin-containing serum, *i.e.* inactivated immune typhoid serum.

C = Complement from the guinea-pig.

M = Mid-piece from guinea-pig complement, corresponding to complement 1 in 10.

E = End-piece from guinea-pig complement, and corresponding to complement diluted 1 in 10.

Mid-piece and End-piece acting independently.

(Illustrative Experiments: See Table I, Exps. 1, 2, 7, 13, 14, 17, 19, 20, 21, 24 and 27.)

The investigation of the normal opsonic amboceptor in guinea-pig serum or in its mid-piece and end-piece fractions did not come specially within the scope of the present research. In the course of our experiments, however, and especially in the earlier ones, it was essential for control purposes to determine the independent action of these fractions.

It will be seen from the experiments in which separate estimations of mid-piece and end-piece are made and compared with complement diluted 1 in 10 that (1) the mid-piece has practically no sensitising action by itself, and (2) the end-piece though possessing a slight though definite action comes very short of that obtained with the diluted complement.

In Exp. 17, it appears that three volumes of end-piece are required to give the same phagocytic index as one volume of complement diluted 1 in 10.

In the two earlier experiments (1 and 2), the mid-piece appears to have a slight action by itself but the exact relation of the mid-piece concentration to that of the complement from which it was precipitated was not known. In all the later experiments the precipitated mid-piece was dissolved in a bulk of saline equivalent to the amount of diluted serum used.

In Exp. 21, the action of four different end-pieces of various ages are compared and on the whole there appears to be little difference between them as estimated by the phagocytic index.

TABLE I. *Phagocytosis Experiments I—XXIX.*

Exp. I. 11/5/11.			Exp. II. 12/5/11.			Exp. III. 15/5/11.		
No previous sensitisation. Age of fractions 2 days. Incubation 15 mins.			No previous sensitisation. Mid-piece prepared 24/3/11 in powder form. Strength of M relative to C unknown.			No previous sensitisation. Same fractions as in Exp. II. Incubation 10 mins.		
	P. I.			P. I.			P. I.	
T (1 in 5) ...	1.3		T (1 in 5) ...	3.0		T (1 in 5) ...	0.3	
Do. + C (1 in 1) ...	3.8		Do. + C (1 in 1) ...	9.1		Do. + C (1 in 1) ...	1.4	
Do. + C (1 in 10) ...	2.3		Do. + M (1 in 5) ...	3.7		Do. + M (1 in 5) ...	0.4	
Do. + M (concentd) ...	0.14		Do. + E (1 in 10) ...	3.6		Do. + E (=1 in 10) ...	0.2	
Do. + M (1 in 10) ...	0.4		C (1 in 1) ...	3.5		C (1 in 1) ...	0.5	
Do. + E (=1 in 10) ...	0.8		M (1 in 5) ...	0.04		Do. + (M ₁ + E ₄) ...	0.4	
C (1 in 1) ...	1.4		E (=1 in 10) ...	0.058				
C (1 in 10) ...	0.8		Heated normal rabbit ser.	0.03				
M (concentd) ...	0.1		Fractions active haemolytically.					
M (1 in 10) ...	0.06							
E (=1 in 10) ...	0.03							
Heated normal rabbit ser.	0.03							
Exp. IV. 17/5/11.			Exp. V. 23/5/11.			Exp. VI. 24/5/11.		
Previous sensitisation. Fractions freshly prepared. Incubation 10 mins.			Previous sensitisation. New E 7 days old. Old E 2 months old. M 7 days old. Incubation 12 mins.			Previous sensitisation. M from powder of 23/3/11. E also of 23/3/11. Incubation 12 mins.		
	P. I.			P. I.			P. I.	
T (1 in 1) ...	0.4		T (1 in 20) ...	0.3		T (1 in 500) ...	3.8	
Do. + C (1 in 10) ...	1.9		Do. + C (1 in 10) ...	2.1		Do. + C (1 in 10) ...	10.8	
Do. + M ...	0.4		Do. + M ...	0.53		Do. + M + 1 E ...	5.8	
Do. + E ...	0.33		Do. + E (new) ...	0.55		Do. + M + 2 E ...	7.8	
Do. + M + E ...	0.53		Do. + E (old) ...	0.58		Do. + M + 3 E ...	8.0	
C (1 in 10) ...	0.009		Do. + M + E (new) ...	0.64		Fractions inactive haemolytically.		
Fractions active haemolytically.			Do. + M + E (old) ...	1.3				
			C (1 in 10) ...	0.07				
			Fractions inactive haemolytically.					
Exp. VII. 29/5/11.			Exp. VIII. 30/5/11.			Exp. IX. 1/6/11.		
Previous sensitisation. Fractions freshly prepared. Incubation 12 mins.			Previous sensitisation. M and E 1 day old. Incubation 12 mins.			Previous sensitisation. Fractions fresh. Incubation 12 mins.		
	P. I.			P. I.			P. I.	
T (1 in 500) ...	1.7		T (1 in 500) ...	2.3		T (1 in 500) ...	3.5	
Do. + 1 C (1 in 10) ...	5.2		Do. + 1 C (1 in 10) ...	12.5		Do. + 1 C (1 in 10) ...	6.3	
Do. + 3 C (1 in 10) ...	5.2		Do. + 3 C (1 in 10) ...	13.1		Do. + 3 C (1 in 10) ...	9.3	
Do. + M ...	1.78		Do. + 3 E ...	5.3		Do. + 1 E ...	3.3	
Do. + 1 E ...	2.3		Do. + M ...	1.3		Do. + 3 E ...	4.7	
Do. + 3 E ...	4.0		Do. + M + 3 E ...	8.5		Do. + M (1 in 10) ...	2.2	
Do. + M + 1 E ...	6.1		E now haemolytically pure.			Do. + do. + 1 E ...	4.9	
Do. + M + 3 E ...	7.1					Do. + do. + 3 E ...	9.4	
1 C (1 in 10) ...	?					Do. + M (1 in 40) ...	4.8	
3 C (1 in 10) ...	1.0					Do. + do. + 1 E ...	7.0	
1 E ...	0.01					Do. + do. + 3 E ...	8.9	
3 E ...	0.6					Do. + M (1 in 160) ...	2.2	
M ...	0.0					Do. + do. + 1 E ...	3.6	
M + 1 E ...	0.09					Do. + do. + 3 E ...	5.6	
M + 3 E ...	1.2					E haemolytically pure.		
Haemolytically E was not pure.								

TABLE I (*continued*).

Exp. X. 6/6/11.			Exp. XI. 3/7/11.			Exp. XII. 4/7/11.		
Previous sensitisation. Same fractions as in Exp. IX, <i>i.e.</i> 5 days old. Incubation 12 mins.			Previous sensitisation. Fractions freshly prepared. Incubation 15 mins.			Previous sensitisation. M of Exp. XI. E 6 days old. Incubation 12 mins.		
		P. I.			P. I.			P. I.
T (1 in 5000) ...	...	0.01	T (1 in 500) ...	...	3.9	T (1 in 500) ...	...	2.6
Do. + 1 C (1 in 10) ...	...	1.16	Do. + C (1 in 10) ...	...	5.6	Do. + C (1 in 10) ...	...	1.7
Do. + 3 C (1 in 10) ...	...	3.7	Do. + M ...	...	0.77	Do. + M ...	...	0.5
Do. + M + 1 E ...	...	0.03	Do. + E ...	...	6.7	Do. + E ...	...	1.5
Do. + M + 3 E ...	...	0.31	Do. + M + E ...	...	2.7	Do. + M + E ...	...	0.3
1 C (1 in 10) ...	...	0.009	Do. + M + C ...	...	5.8	Do. + M + C ...	...	1.4
3 C (1 in 10) ...	...	1.10	C (1 in 10) ...	...	0.0	Do. + heated M ...	...	1.9
			E pure haemolytically.			Do. + do. + E ...	...	0.6
Exp. XIII. 18/7/11.			Exp. XIV. 19/7/11.			Exp. XV. 20/7/11.		
Previous sensitisation. M and E, 8 days old. Incubation 13 mins.			Previous sensitisation. Fractions same as in Exp. XIII, <i>i.e.</i> 9 days old. Incubation 14 mins.			Previous sensitisation. Fractions same as in Exp. XIII, <i>i.e.</i> 10 days old. Incubation 13 mins.		
		P. I.			P. I.			P. I.
T (1 in 500) ...	...	1.8	T (1 in 500) ...	...	1.7	T (1 in 500) ...	...	2.5
Do. + C (1 in 5) ...	...	2.5	Do. + C (1 in 5) ...	...	5.0	Do. + C (1 in 5) ...	...	0.67
Do. + M ...	...	0.12	Do. + M ...	...	0.01	Do. + M ...	...	0.18
Do. + 3 E ...	...	4.3	Do. + 3 E ...	...	2.4	Do. + 3 E ...	...	4.2
Do. + M + 3 E ...	...	4.4	Do. + M + 3 E ...	...	0.6	Do. + M + 3 E ...	...	0.59
Do. + M + 3 heated E ...	...	1.8	Do. + M + 3 heated E ...	...	2.5	Do. + M + 3 heated E ...	...	0.34
Do. + M + C (1 in 5) ...	...	5.8	Do. + M + C (1 in 5) ...	...	5.4	Do. + M + C (1 in 5) ...	...	1.1
C (1 in 5) ...	...	0.12	C (1 in 5) ...	...	0.12			
3 E ...	...	0.01	3 E ...	...	0.009			
Exp. XVI. 21/7/11.			Exp. XVII. 21/9/11.			Exp. XVIII. 22/9/11.		
Previous sensitisation. Fractions same as in Exp. XIII, <i>i.e.</i> 11 days old. Incubation 13 mins.			Previous sensitisation. Fractions freshly prepared. E heated $\frac{1}{2}$ hour at 56° C. Incubation 12 mins.			Previous sensitisation. Same fractions, <i>i.e.</i> 1 day old. Incubation 12 mins.		
		P. I.			P. I.			P. I.
T (1 in 500) ...	...	1.2	T (1 in 500) ...	...	0.5	T (1 in 500) ...	...	2.2
Do. + 1 C (1 in 5) ...	...	4.7	Do. + 2 C (1 in 10) ...	...	0.54	T (1 in 10) ...	...	0.77
Do. + 3 C (1 in 5) ...	...	5.8	Do. + M ...	...	0.26	T (1 in 500) + M ...	...	0.05
Do. + 3 E ...	...	4.1	Do. + 3 E ...	...	0.87	T (1 in 10) + M ...	...	0.39
Do. + 3 heated E ...	...	2.9	Do. + 3 heated E ...	...	1.01	T (1 in 500) + 3 E ...	...	2.1
Do. + M + 3 E ...	...	1.0	Do. + M + 3 E ...	...	1.3	T (1 in 500) + 3 heated E ...	...	1.4
Do. + M + 3 heated E ...	...	0.87	Do. + M + 3 heated E ...	...	1.09	T (1 in 10) + 3 E ...	...	0.55
Do. + M + C (1 in 5) ...	...	6.8	1 C (1 in 10) ...	...	0.14	T (1 in 10) + 3 heated E ...	...	1.3
			3 C (1 in 10) ...	...	1.06	T (1 in 500) + M + 3 E ...	...	0.14
			3 E ...	...	0.10	T (1 in 500) + M + 3 heated E ...	...	0.04
			3 heated E ...	...	0.01	T (1 in 10) + M + 3 E ...	...	0.72
						T (1 in 10) + M + 3 heated E ...	...	0.77
Exp. XIX. 26/9/11.			Exp. XX. 27/9/11.			Exp. XXI. 29/7/11.		
Previous sensitisation. Same fractions, <i>i.e.</i> 5 days old.			Previous sensitisation. Same fractions, <i>i.e.</i> 6 days old. Incubation 12 mins.			Previous sensitisation. End-pieces of various ages. Incubation 12 mins.		
		P. I.			P. I.			P. I.
T (1 in 500) ...	...	3.0	T (1 in 500) ...	...	5.0	T (1 in 500) ...	...	1.7
Do. + M ...	...	0.22	Do. + M ...	...	0.28	Do. + 3 E "a" (1 day old) ...	...	3.5
Do. + 3 E ...	...	1.5	Do. + 3 E ...	...	4.9	Do. + 3 E "β" (Fresh) ...	...	2.7
Do. + M + 3 E ...	...	1.6	Do. + M + 3 E ...	...	4.8	Do. + 3 E "γ" (Fresh) ...	...	1.3
3 E ...	...	0.09	3 E ...	...	0.0	Do. + 3 E "δ" (10 days) ...	...	1.8
						3 E "a" ...	...	0.007
						3 E "β" ...	...	0.02
						3 E "γ" ...	...	0.03
						3 E "δ" ...	...	0.03

TABLE I (*continued*).

Exp. XXII. 4/10/11.		Exp. XXIII. 9/10/11.		Exp. XXIV. 20/10/11.	
Previous sensitisation. Effect of filtered mid-pieces of various ages. Incubation 12 mins.		Previous sensitisation. Fractions freshly prepared. Mid-piece filtered through paper. Incubation 12 mins.		Previous sensitisation. Fractions freshly prepared. Incubation 14 mins.	
	P. I.		P. I.		P. I.
T (1 in 500) ...	0.30	T (1 in 500) ...	3.3	T (1 in 200) ...	9.5
Do. + M "a" (16 days)...	0.29	Do. + M ...	0.4	Do. + M ...	1.9
Do. + M "b" (14 days)...	0.21	Do. + 3 E... ..	5.9	Do. + 3 C (1 in 10)...	11.6
Do. + M "c" (6 days) ...	0.24	Do. + M + 3 E	5.4	Do. + 3 E ..	7.2
Do. + M "d" (6 days) ...	0.22	Fractions active haemolytically.		Do. + 3 heated E ...	7.3
Do. + M "e" (2 days) ...	0.34			Do. + M + 3 E ...	9.8
Do. + M "f" (Fresh) ...	0.10			Do. + M + 3 heated E ...	1.8
Do. + 3 E... ..	0.77			Do. + M + 3 C (1 in 10) ...	8.3
Do. + M "a" + 3 E ...	2.2			Do. + heated M ...	7.4
Do. + M "b" + 3 E ...	1.1			Do. + heated M + 3 E ...	7.3
Do. + M "c" + 3 E ...	1.3			Do. + heated M + 3 heated E ...	8.8
Do. + M "d" + 3 E ...	2.7			Do. + heated M + 3 C (1 in 10) ...	8.5
Do. + M "e" + 3 E ...	2.7			3 C (1 in 10) ...	0.12
Do. + M "f" + 3 E ...	3.3			3 E ...	0.0
				3 heated E ...	0.0
Exp. XXV. 21/10/11.		Exp. XXVI. 22/10/11.		Exp. XXVII. 6/11/11.	
Previous sensitisation. Same fractions, <i>i.e.</i> 1 day old. Incubation 12 mins.		Previous sensitisation. Effect of filtered, unfiltered and heated mid-pieces.		Fractions freshly prepared. Incubation 14 mins.	
	P. I.		P. I.		P. I.
T (1 in 500) ...	5.4	T (1 in 500) ...	4.7	T (1 in 500) ...	4.1
Do. + M (1 in 10) ...	0.3	Do. + filtered M "a" ...	0.52	Do. + M (1 in 10) ...	5.2
Do. + M (1 in 20) ...	1.1	Do. + unfiltered M "a" ...	0.14	Do. + M (1 in 20) ...	4.2
Do. + M (1 in 40) ...	1.3	Do. + heated M "a" ...	3.7	Do. + M (1 in 40) ...	4.1
Do. + heated M ...	4.9	Do. + 3 E... ..	3.4	Do. + M (1 in 80) ...	3.0
Do. + 3 E ...	4.3	Do. + filtered M "a" + 3 E	0.6	Do. + 3 E ...	5.2
Do. + M (1 in 10) + 3 E...	0.7	Do. + unfiltered M "a" + 3 E	2.6	Do. + M (1 in 10) + 3 E...	8.1
Do. + M (1 in 20) + 3 E...	0.38	Do. + heated M "a" + 3 E	1.9	Do. + M (1 in 20) + 3 E...	7.6
Do. + M (1 in 40) + 3 E...	0.5	Do. + filtered M "b" ...	4.6	Do. + M (1 in 40) + 3 E...	6.9
Do. + heated M + 3 E ...	1.1	Do. + unfiltered M "b" ...	2.1	Do. + M (1 in 80) + 3 E...	5.1
		Do. + heated M "b" ...	5.9	Do. + 3 C (1 in 10) ...	8.6
		Do. + filtered M "b" + 3 E	2.0	3 C (1 in 10) ...	1.1
		Do. + unfiltered M "b" + 3 E	2.9	3 E ...	0.008
		Do. + heated M "b" + 3 E	2.4		
Exp. XXVIII. 8/11/11.		Exp. XXIX. 10/11/11.			
Same fractions, <i>i.e.</i> 2 days old.		Previous sensitisation. Same fractions, <i>i.e.</i> 4 days old.			
	P. I.		P. I.		
T (1 in 500) ...	5.8	T (1 in 500) ...	4.3		
Do. + M (1 in 10) ...	2.9	Do. + M (1 in 10) ...	0.46		
Do. + M (1 in 20) ...	5.3	Do. + M (1 in 20) ...	2.02		
Do. + 3 E... ..	6.7	Do. + M (1 in 10) + 3 E...	1.4		
Do. + 3 heated E ...	8.1	Do. + M (1 in 20) + 3 E...	3.7		
Do. + M (1 in 10) + 3 E...	11.0				
Do. + M (1 in 20) + 3 E...	12.3				
Do. + M (1 in 10) + 3 heated E	7.4				

The end-piece when inactivated still possesses a very slight action (see Exp. 17). The independent action of end-piece though admittedly slight is best explained on the assumption that small amounts of active mid-piece are still present in the filtrate after removal of the deposit, and although in the haemolytic system (Part II) these fractions appeared to be pure, it is possible that, in a phagocytosis system, minute traces of mid-piece in the end-piece may be put in evidence. Moreover from certain results to be detailed later it would appear that the removal of the mid-piece may in certain cases enhance markedly the value of the end-piece relatively to the untreated serum.

The Action of Mid-piece and End-piece in concert.

On this point Exp. 7, may be consulted. In the latter part of that experiment, values of mid-piece and end-piece and combinations of such are compared with those of the diluted complement. It will be seen that three volumes of end-piece in combination with mid-piece give practically the same value as three volumes of complement diluted 1 in 10. Without the addition of mid-piece the three volumes of end-piece give only half the value of the diluted complement. It has to be noted, however, that this particular experiment was one of the few in which the mid-piece exerted no inhibitory action throughout and consequently excellent complementing effects ensued.

Hata (1908) in a communication on the structure of the normal opsonin performed a few opsonic experiments with staphylococci and the fractions of normal guinea-pig serum obtained by the method of dialysis. Neither of the fractions appeared to have any considerable sensitising power when acting independently, but when acting in concert, values were obtained which approached far more closely to those given by the original serum than we were able to obtain in our experiments.

The Action of Complement on Tropin.

It is unnecessary to discuss this question in any detail as it has been shown by various workers that an inactivated immune typhoid serum which of itself effects sensitisation of organisms for phagocytosis (so-called tropin-content) can have this action greatly enhanced by the addition of guinea-pig complement. In the great majority of the experiments recorded in the tables, such complementing effects are very clearly seen, the total effect of the mixture of heated immune serum (T)

and complement being considerably greater than the sum of the two separate effects. In a few experiments, however (*e.g.* Exps. 12, 15, 17), no complementing effect occurred. In fact the value of the mixture was (in two of these experiments) considerably less than that of the tropin acting separately. In some of these experiments the complement though inactive or inhibitory in the phagocytosis system was found to be active in a haemolytic system.

This peculiar action of complement on tropin cannot be readily explained without examining a large series of guinea-pig complements. Out of over 20 complements tested it occurred only three times. From the frequent inhibitory effects obtained with various mid-pieces and presently to be described, it might be suggested that in a particular complement the ratio of mid-piece to end-piece may be altered, with the result that inhibition of the tropic effect occurs owing to excess of mid-piece. The fact that in the haemolytic system such complements proved active is of great interest, but it has to be remembered that in the latter case the time factor may be of importance. In the phagocytosis experiments the period of incubation could not be extended much beyond 12 minutes owing to the onset of intracellular lysis which affected considerably the accuracy of the count. It is also not unlikely that the inactivity of the complement in these few cases may have been due to a slight cloudiness not observed at the time of experiment.

Remarks on Tropins and Immune Opsonins.

Our experiments were not directly devised with a view to obtaining evidence on the identity or non-identity of the tropins and immune opsonins, as we are of opinion that the evidence drawn simply from their mode of action cannot be employed in support of the view that these substances are essentially different. In Exp. 10 an attempt was made to determine whether the addition of complement to an inactivated immune serum diluted to a point where tropic action was in abeyance, would still give a definite complementing result. It will be seen from this experiment that a very definite complementing effect was obtained, a result which we are inclined to regard as analogous to the marked fixation of complement which occurs in mixtures of antigen and antibody which yield no visible precipitate. In this connection the recent experiments of Dean (1911) may be consulted.

By adding together the separate values of tropin and complement and subtracting the sum from that obtained with the mixture of tropin

and complement, the percentage increase so calculated may give some measure of the fixation of complement in each case. This varies very greatly as might be expected in the different experiments. In Exps. 1, 2, 3, 4, 5, 7, 10, 11, 13, 14, 24 and 27 the percentage increases are respectively: 40.7%, 40%, 75%, 364%, 467%, 92%, 233%, 43%, 30.2%, 174%, 20.5%, and 65.3%.

We do not attach much importance to these figures as the conditions in the different experiments were not strictly comparable. It is of interest, however, that Exp. 24, in which the mixtures of antigen and antibody employed gave rise to a very high tropin effect, showed the least complementing effect—a result which may be compared with the insignificant fixation of complement which occurs in mixtures of antigen and antibody which give rise to large visible precipitates. We put forward these analogies here solely with the view of directing attention to the possibility that the union of antigen and antibody may give rise to similar redistribution of the constituent colloid particles of the system, the effects of which will vary according to the particular system of investigation employed (precipitating, tropic, agglutinative, etc.).

*The Action of Mid-piece on Tropin and the Effect of final
addition of End-piece.*

Practically all the experiments recorded in the tables contain evidence on this question, but attention will be directed to particular groups of these in which the addition of mid-piece to tropin resulted uniformly in an inhibition of the latter or was indifferent.

We shall consider in the first place those experiments in which the addition of mid-piece (corresponding to complement diluted 1 in 10) depresses the tropic action (see Table I, Exps. 1, 8, 9, 11, 12, 13, 14, 15, 17, 18, 19, 20, 22, 23, 24, 25, 26 and 29). The percentage degrees of inhibition calculated from these figures are respectively:

- | | | | | | |
|----------|----------|----------------|--|-------------------------|-----------------|
| (1) 69% | (8) 43% | (9) 37%
37% | (11) 80% | (12) 80% | (13) 93% |
| (14) 99% | (15) 92% | (17) 48% | (18) 97% (T 1 in 500)
49% (T 1 in 10) | (19) 92% | (20) 94% |
| (22) 66% | (23) 87% | (24) 80% | (25) 92%
79%
75% | (26) 88%
97%
2.1% | (29) 89%
53% |

(Degrees of depression under 25% have been reckoned under the category of "Indifferent" (see Table II)).

It will be seen that the addition of mid-piece causes in a very large proportion of the experiments a marked depression of the tropic value as estimated by the phagocytic index. In the annexed table (Table II) data bearing on this point have been extracted from the various

TABLE II.

Exp.	Effect of mid-piece		Effect of final addition of end-piece		
	Depressant	Indifferent	Depressant	Indifferent	Complementing
5	...	+	...	...	+
7	...	+	...	...	+
9 (2)	...	+	...	...	+
22 (a)	...	+	...	...	+
22 (c)	...	+	...	...	+
22 (d)	...	+	...	...	+
22 (e)	...	+	...	...	+
27 (1)	...	+	...	...	+
27 (2)	...	+	...	...	+
27 (3)	...	+	...	...	+
28 (2)	...	+	...	...	+
27 (4)	...	+	...	+	...
3	...	+	...	+	...
4	...	+	...	+	...
26 (3)	...	+	+	...	...
22 (6)	32 $\frac{0}{10}$	...	...	...	+
9 (1)	37	...	...	...	+
9 (3)	37	...	...	+	...
8	43	...	...	...	+
17	48	...	...	...	+
T (1 in 10) 18	49	...	...	...	+
28 (1)	50	...	...	...	+
29 (2)	53	...	...	+	...
26 (4)	55	...	...	+	...
22 (f)	66	...	...	...	+
M (1 in 40) 25	75	...	+	...	...
M (1 in 20) 25	79	...	+	...	...
24	80	...	...	+	...
11	80	...	+	...	...
12	80	...	+	...	...
23	87	...	...	+	...
26 (1)	88	...	+	...	...
29 (1)	89	...	+	...	...
15	92	...	+	...	...
19	92	...	+	...	...
M (1 in 10) 25	94	...	+	...	...
13	93	...	...	+	...
20	94	...	...	+	...
T (1 in 500) 18	97	...	+	...	...
26 (2)	97	...	+	...	...
14	99	...	+	...	...

experiments and arranged in such a manner as to show in order the effects of the various mid-pieces tested, from "indifferent" action to ascending degrees of inhibition. In this table the effects of final addition of the end-piece are also included and it will be apparent that a definite relationship exists between these two effects.

If the mid-piece is "indifferent," *i.e.* if the tropic value remains practically unaltered by addition of mid-piece, the subsequent addition of end-piece gives, as a rule, a marked complementing effect, *i.e.* the value of the mixture $T + M + E$ is very much greater than the value of $T + E$. Even in those instances in which the mid-piece depresses the tropic value by amounts up to 50% the addition of end-piece is still able to cause definite complementing action. Greater degrees of inhibition by mid-piece cannot, however, be overcome by addition of end-piece, and the final value of $T + M + E$ is either "indifferent," *i.e.* not greater than $T + E$, or is considerably less than $T + E$ ("depressant"). The fact that the end-piece can complement a system in which the persensitised organisms possess a marked degree of negative tropism towards the leucocyte, raises the question of the exact nature of persensitisation from the colloidal point of view. Leaving aside for the moment the consideration of the factors which determine the inhibitory or non-inhibitory character of a certain mid-piece solution, let us examine the probable action of mid-piece when in contact with a tropin solution and bacilli. The tropic substances of the immune serum are, we presume, associated with the end-piece fraction of that immune serum. That this is so, will be shown later.

The mid-piece may be inhibitory in three ways:

- (1) by partial absorption of the immune end-piece thus preventing the bacilli from being efficiently sensitised by the tropin;
- (2) by acting on the bacilli forming a protective coating on them and so preventing the action of the tropic substances;
- (3) by some inhibitory action on the leucocytes.

We have tested both the second and third hypotheses and found that it makes no difference whether the mid-piece be added in the first instance to the bacilli and digested for a time with them, the tropin being added later, or whether the mid-piece is added subsequently to the tropin. Also, so far as we have observed the leucocytes do not appear to have their phagocytic powers affected by being digested in a mid-piece solution. There remains the first hypothesis which we think will largely explain the facts. We have observed that when the tropin is used in high concentration, the addition of mid-piece does not have

so great an inhibitory effect as when great dilution of tropin is used (see Exp. 18). Other experiments bearing on this point will be described later. It seems most probable, therefore, that *inhibition by mid-piece* when it does occur, is due to partial absorption of the immune end-piece, thus preventing the primary sensitisation of the organisms by the tropin. It will be shown in the haemolytic experiments (see Part II) that an old mid-piece has a distinct inhibitory action on the end-piece of the normal complement. This action in the case of the phagocytosis experiments may contribute to the depressant effect in those cases in which final addition of end-piece was made to a mixture of tropin and mid-piece which was itself inhibitory.

Action of End-piece on Tropin.

As we have already mentioned, the end-piece as separated by the CO_2 method has a slight though definite action by itself, doubtless owing to the presence of small quantities of mid-piece. When added to tropin, this end-piece had, in a large number of cases, a complementing action on tropin in the absence of a further supply of mid-piece. In several cases, haemolytic experiments were performed on the same day with the same materials and not infrequently it was found that a marked complementing action by the end-piece itself in the phagocytosis system coincided with a similar independent though slight lytic action of the end-piece in the haemolytic system. In other cases the end-piece for lytic purposes was quite inactive while still possessing considerable complementing properties in the phagocytosis system.

Of 32 experiments bearing on this question the end-piece had a complementing action in 16 cases (+ 25 % to + 240 %), a depressant action in six cases (− 25 % to − 50 %) and an indifferent action in ten cases (− 25 % to + 25 %).

Of 13 experiments in which the end-piece was complementary by itself, further complementing was obtained in the system $T + M + E$ in eight cases. In the remaining five cases the mid-piece was so inhibitory as to overcome any complementing action that the end-piece may have possessed in the absence of mid-piece.

The conclusion is clear from the analysis of the various experiments that definite complementing effects are to be obtained in the system $T + M + E$ only when M possesses by itself an indifferent or a slightly depressant action on T . With higher degrees of depression, the end-piece is unable to convert the system $T + M$ from a “negatively tropic” to a “positively tropic” state.

Action of Mid-piece on a Mixture of Tropin and Complement.

(Table I, Exps. 11, 12, 13, 14, 15, 16, 24.)

It is of some interest that the complementing action of complement on tropin is often markedly enhanced when mid-piece is present in the mixture, even though the particular mid-piece in combination with tropin causes great inhibition. (Compare haemolytic experiments on this point, Part II.)

Effect of keeping on the Mid-piece and End-piece and suggested explanation of inhibitory phenomena, with the comparative haemolytic experiments.

It will be seen from the tables that there is a tendency for a mid-piece, which when fresh exerts no inhibitory action on tropin, to become very markedly inhibitory on keeping (see particularly Exps. 27, 28 and 29 in which the same materials were employed on different dates). On the first day the mid-piece was not inhibitory in a dilution of 1 in 10. On the second day this dilution had a depressant action of 50%, while on the third day the depressant action was as high as 89% and the dilution of 1 in 20 was also inhibitory to the extent of 53%. It was observed that in the cold room the mid-piece solution tended to become cloudier on standing, and this, as we have seen, was accompanied by increased inhibitory powers. None the less it frequently happened that a freshly prepared and apparently clear mid-piece possessed definite inhibitory properties in dilution of 1 in 10.

It is obvious that the physical state of the colloid particles of the mid-piece solution plays a great part in determining its indifferent action on tropin and its action on the joint system T + M + E (see Exp. 26). It is of interest to compare with this inhibition effect the inhibitions got by Schmidt (1911) in cloudy globulin solutions in elucidating the Wassermann reaction on colloidal principles.

The end-piece on the other hand does not appear to depreciate on standing and excellent results have been obtained with end-pieces which have been kept for over two months in the cold room. Mid-piece solutions which have been passed through a Berkefeld filter lose their inhibitory power as well as their capacity for complementing in conjunction with end-piece. (Compare haemolytic experiments on this point, Part II.)

Effect of Heated Mid-piece and End-piece on Tropin.

Heating the mid-piece for one hour at 55° C. entirely removes any inhibitory action it may have had on tropin independently (see Exps. 12, 24, 25 and 26). In two of these experiments the following values were obtained:

T	=	5.4	T	=	9.5
T + M (1 in 10)	=	0.3	T + M	=	1.9
T + M (1 in 20)	=	1.1	T + heated mid-piece	=	7.4
T + M (1 in 40)	=	1.3	T + heated M + E	=	7.3
T + heated mid-piece	=	4.9			
T + heated M + E	=	1.1			

In none of these experiments with heated mid-piece however was a complementing effect obtained on subsequent addition of end-piece. In fact the value of the combination T + heated M + E was in two cases much less than that of T + E in spite of the indifferent action of the heated mid-piece on tropin. It is suggested therefore that the heated mid-piece has no absorbing action on the immune rabbit end-piece containing the tropin but a very marked absorbent action on the guinea-pig end-piece.

The end-piece does not appear to be so greatly influenced by heat (see Exps. 13, 14, 15, 16, 17, 18, 24, and 28) as the mid-piece when its action on tropin is investigated.

It would appear that the single addition of heated end-piece to tropin may in some cases increase the tropin value, but as a rule not to the same degree as the unheated end-piece. Occasionally however the heated end-piece has even exceeded the unheated end-piece in this action. In the presence of an inhibitory mid-piece the heated end-piece has in nearly all cases less power than the unheated end-piece has, of converting the "negatively tropic" system T + M. Experiment 24 is an interesting illustration of this point. The mid-piece in this case was very markedly inhibitory and the value of T compared with that of T + M was as 9.5 to 1.9 (5:1). The addition of end-piece to the system T + M had just the effect of restoring the initial conditions (T + M + E = 9.8), whereas the addition of heated end-piece to the system T + M brought about no increase (T + M + heated E = 1.8).

Further experiments however will require to be performed before we can draw any definite conclusions regarding the thermo-resistance of the end-piece in relation to a phagocytic system.

Experiments with Mid-piece and End-piece obtained from Rabbit Complement.

In order to ascertain whether similar effects were to be obtained with the complement-fractions obtained from the rabbit the following experiments were performed.

Experiment I. 27. x. 11.

R.E. = Rabbit end-piece. R.M. = Rabbit mid-piece. G.M. = Guinea-pig mid-piece.
G.E. = Guinea-pig end-piece. R.C. = Rabbit complement.

Fractions freshly prepared.

				Phagocytic index
T (1 in 500)	...	...	...	9·6
Do. + R.C.	...	...	...	22·7
Do. + R.E.	...	...	...	10·9
Do. + R.M.	...	...	...	2·4
Do. + heated R.M.	...	...	...	10·9
Do. + G.E.	...	...	...	10·3
Do. + heated R.E.	...	...	...	9·5
Do. + R.M. + R.E.	...	...	...	16·8
Do. + R.M. + G.E.	...	...	...	7·4
Do. + heated R.M. + R.E.	...	...	...	17·0
R.C.	...	...	...	1·8
R.E.	...	...	...	0·01
G.E.	...	...	...	0·0

It will be seen that the rabbit mid-piece was inhibitory to tropin but that the heated rabbit mid-piece was quite indifferent.

Good complementing was obtained on final addition of rabbit end-piece, but the effect of guinea-pig end-piece was only to neutralise the inhibitory effect of the rabbit mid-piece.

The rabbit complement gave excellent complementing with tropin.

Good complementing was also obtained with rabbit end-piece in the presence of heated rabbit mid-piece so that from this experiment one might assume a considerable resistance to heat on the part of the rabbit mid-piece.

A similar experiment on 28th October, 1911, with the same materials gave the following result :

				Phagocytic index
T (1 in 500)	...	...	...	5·0
Do. + R.E.	...	...	...	7·6
Do. + R.M.	...	...	...	0·15
Do. + R.M. + R.E.	...	...	...	8·2

It will be seen that the inhibition by rabbit mid-piece amounted to 97 % (75 % on previous day) on this occasion and that little or no complementing ensued on addition of the rabbit end-piece.

A later experiment on 31st October, 1911, was made with the same fractions (now five days old).

	Phagocytic index		
T (1 in 1000)	...	...	2.2
Do. + R.E.	...	...	3.3
Do. + R.M.	...	...	0.16
Do. + R.M. + R.E.	...	...	2.0
Do. + heated R.M.	...	...	3.4

Again the rabbit mid-piece was very inhibitory (92 %) and no complementing occurred with rabbit end-piece.

Experiment.

2. xi. 11. On this date an attempt was made to compare the efficiency of complement-fractions from the guinea-pig with those from the rabbit. Fresh rabbit and guinea-pig sera were split on the same day. The tropin was used in a dilution of 1 in 1000 and 14 minutes incubation were given.

Result :

	Phagocytic index		
T (1 in 1000)	...	...	3.1
A. Do. + R.E.	...	...	2.4
Do. + G.E.	...	...	4.5
B. Do. + R.M.	...	...	1.0
Do. + G.M.	...	...	1.5
C. Do. + R.C.	...	...	6.6
Do. + G.C.	...	...	4.6
D. R.C.	...	...	2.0
G.C.	...	...	1.7
E. Do. + R.M. + R.E.	...	...	1.9
Do. + R.M. + G.E.	...	...	3.1
F. Do. + G.M. + G.E.	...	...	4.0
Do. + G.M. + R.E.	...	...	3.8
G. Do. + R.M. + R.C.	...	...	7.2
Do. + R.M. + G.C.	...	...	3.8
H. Do. + G.M. + G.C.	...	...	6.2
Do. + G.M. + R.C.	...	...	10.6

Guinea-pig mid-piece appears on the whole to act better than rabbit mid-piece in the presence both of guinea-pig end-piece and rabbit end-piece, *i.e.* the rabbit end-piece goes better with the guinea-pig mid-piece than with the homologous mid-piece. Also as in the previous experiments the rabbit mid-piece went better with the homologous end-piece than with the guinea-pig end-piece, *i.e.*

$$R.E. + G.M. > R.E. + R.M. > G.E. + R.M.$$

As in previous experiments the addition of mid-piece in the system T + C increases the complementing action, and in this respect the guinea-pig mid-piece is again more powerful than the rabbit mid-piece.

It cannot be said, however, that either the rabbit end-piece or the rabbit complement is at all inferior to guinea-pig end-piece or guinea-pig complement when a suitable mid-piece (guinea-pig) is supplied.

The haemolytic experiments performed in this connection (see Part II) do not bear out this result. In fact the latter show very clearly that rabbit complement contains as efficient a mid-piece as guinea-pig complement but that its end-piece has practically no lytic action with either mid-piece. It would be necessary to extend the phagocytosis experiments considerably before concluding that this difference in the effects got by the two systems is a valid one. It may be that the proportions of mid-piece and end-piece necessary for complementing a tropin system differ considerably from those necessary for lysis. The suggestion has occurred to us that the concentration of mid-piece employed by us in the majority of the experiments may have been too high, and that in fact the amount of mid-piece left in the supernatant fluid with the end-piece, after saturation with CO_2 , may be sufficient in many cases for complementing purposes. We hope to make further experiments in this connection.

Effect of splitting the Immune Tropin-containing Serum with CO_2 .

For this purpose an immune serum (to *B. typhosus*) was prepared from another rabbit and immediately after bleeding, saturated with CO_2 in the usual way. Care was taken to render the end-piece filtrate as free of mid-piece as possible by prolonged sedimentation after centrifugalisation.

Our object was to determine whether some explanation could be afforded of the *lessened inhibitory action* of mid-piece on tropin in high concentration than on highly diluted tropin. It was hoped also that some light might be thrown on the inhibition zone phenomenon so frequently noticed in highly concentrated immune sera.

Experiment I.

Comparison of the tropic values of the ordinary heated immune serum and of the same serum freed of mid-piece. The latter will be called in this and subsequent experiments the "immune end-piece."

Both fluids were digested as usual with the bacillary emulsion for 1 hour at 37° C. before the phagocytosis experiment was made.

Result :

Ordinary immune serum	Phagocytic index	Immune end-piece	Phagocytic index
1 in 10	6.4	1 in 10	18.7
1 in 100	4.6	1 in 100	5.0
1 in 1000	0.12	1 in 1000	0.37
1 in 5000	0.11	1 in 5000	0.12

It will be seen that removal of the mid-piece has increased enormously the tropic value of the immune serum in dilution of 1 in 10. In lower dilutions the effects are not obvious.

Experiment II.

Similar materials employed. Phagocytosis 12 minutes.

Ordinary immune serum	Phagocytic index	Immune end-piece	Phagocytic index
1 in 10	1.8	1 in 10	4.4
1 in 50	5.1	1 in 50	4.9
1 in 100	3.2	1 in 100	5.6

The same phenomenon is again seen in the 1 in 10 dilutions.

Experiment III.

In this experiment the same materials were employed but a comparison was also made with immune end-piece, to various dilutions of which guinea-pig mid-piece was added.

Result :

Ordinary immune serum	Phagocytic index	Immune end-piece	Phagocytic index	Immune end-piece +g.-pig mid-piece	Phagocytic index
1 in 10	8.7	1 in 10	16.1	1 in 10 + M	5.5
1 in 50	11.2	1 in 50	13.5	1 in 50 + M	2.4
1 in 100	8.1	1 in 100	7.6	1 in 100 + M	2.4

Again the immune end-piece in dilution of 1 in 10 is much more powerful than the ordinary immune serum in the same dilution. In lower dilutions the values are equalised. The effect of adding guinea-pig mid-piece to the various dilutions of immune end-piece causes very marked inhibition in all. It would appear, therefore, that to get the full effect of an immune serum in high concentration it is an advantage to get rid of the mid-piece (or the greater part of it).

Experiment IV.

Attempt to ascertain whether the inhibitory effect of the mid-piece was exerted better by keeping it first in contact for a time with the bacilli and then later adding the immune end-piece. Bacilli and mid-piece were digested together for half an hour at 37° C. The immune end-piece was then added (Fluid A). Bacilli and mid-piece and immune end-piece were added together simultaneously and digested for the same time (Fluid B). Bacilli and immune end-piece were digested together (Fluid C). Bacilli and immune end-piece and heated mid-piece were digested together (Fluid D). The dilution of immune end-piece employed was 1 in 50, and the mid-piece was the ordinary guinea-pig mid-piece (= 1 in 10).

Result :

	Phagocytic index
Fluid A	0.26
„ B	0.21
„ C	2.3
„ D	0.84

Apparently therefore there is no difference between A and C. The mid-piece is very inhibitory (88 % reduction). The heated mid-piece has as usual much less inhibitory action (63 %).

Experiments on this subject are being continued.

PART II.

HAEMOLYTIC EXPERIMENTS.

On the Properties of the Separated Fractions of the Complement.

The effect of heat on the two fractions of the complement has been investigated by numerous workers. Ferrata's original communication contains the statement that the globulin fraction withstands a temperature of 55° C. while the so-called albumen fraction is destroyed.

Brand (1907) found that both fractions were thermolabile and this conclusion has been confirmed by Tsurusaki (1908), Sachs and Bolkowska (1910) and Fränkel (1911). Mutermilch (1911) heated fresh guinea-pig serum for half an hour at temperatures varying from 53° C. to 57° C. The heated serum was then dialysed as in Ferrata's method. The time necessary to obtain precipitation of the euglobulin increased with the degree of temperature which had been employed. Serum heated to 53° C. or 54° C. required several hours, serum heated to 55° C. 24 to 48 hours and serum heated to 56° C. or 57° C. commenced to precipitate after seven or eight days. A mixture of the globulin obtained from heated serum with the supernatant fluid obtained by the dialysis

of fresh serum produced haemolysis. Haemolysis was frequently obtained by mixing the supernatant fluid from heated serum with the globulin of fresh serum. Occasionally (in four cases out of twelve) Mutermilch obtained haemolysis by mixing the two fractions obtained from heated serum.

Similar results were obtained by H. R. Marks (1911) who was able to reactivate heated guinea-pig serum by the addition of fresh end-piece. On one occasion a guinea-pig serum which had stood for 17 days at room temperature was reactivated with fresh end-piece. Marks also separated the globulin from heated serum both by Sachs and Altmann's and by Liefmann's method. He was able to reactivate the precipitate so obtained with fresh end-piece.

In considering the properties of preparations of mid-piece and end-piece it is necessary to remember that we are dealing with suspensions of serum proteids in salt solution, and that the properties which can be demonstrated are largely dependent on the somewhat artificial experimental conditions.

The mid-piece preparations obtained in the method described in the first part of the paper were immediately after their preparation perfectly clear. They were kept in the cold room, and after a period varying from 24 hours to six days they began to get turbid and eventually a definite deposit formed at the bottom of the bottles in which they were kept. They remained active for variable periods, usually at least a week and often for as long as six or seven weeks. During this time there was a very gradual deterioration in their haemolytic properties. The activity of an old mid-piece preparation could only be demonstrated by allowing the mid-piece to act for about 20 minutes on a mixture of corpuscles and haemolysin and then adding the end-piece. Fresh mid-piece solutions produced haemolysis when added together with the end-piece to sensitised corpuscles. Better results were, however, obtained even with fresh mid-piece by the preliminary incubation method. This property of the mid-piece will be referred to at length in a subsequent paragraph. If the preliminary incubation method was employed it was often possible to demonstrate the activity of mid-piece solutions many weeks after their preparation.

During the course of these experiments the effect of heat on three different samples was examined. Both fractions were in each case found to be thermolabile. The mid-piece was, however, distinctly more thermolabile than the end-piece.

The phagocytic, haemolytic and inhibitory properties of the mid-piece were readily destroyed by heat. The haemolytic action of the end-piece was destroyed more easily than its phagocytic action.

The results of a typical haemolysis experiment are shown in Table III.

The end-piece preparations in fact showed themselves to be in every way more stable than those of the mid-piece. Preparations were kept for as long as six months in the cold room without apparent loss of haemolytic power.

Another difference between the two fractions was demonstrated by filtration. The mid-piece was inactivated by filtering it through a Berkefeld filter. The end-piece preparation passed through a Berkefeld filter without any loss.

An attempt was made to filter the end-piece solution through a gelatin filter. The filtrate was found to be inactive.

These experiments suggested the possibility of effecting a separation of the complement-fractions by passing guinea-pig serum through a Berkefeld filter. Several specimens of guinea-pig serum were accordingly passed through a Berkefeld filter, but the filtered serum showed no loss of complement.

Explanation of scheme used to illustrate the complement fixation experiments.

No haemolysis	...	...	
Trace	„	...	
Slight	„	...	
Half haemolysed	...	...	
Strong or marked haemolysis			
Almost complete	„	...	
Complete	„	...	

Each tube in the original experiment is represented by a square in the Table. A black square represents total haemolysis. A white square represents a tube in which no haemolysis has occurred.

TABLE III.

To show the effect of heating the separated mid- and end-piece at a temperature of 55° C.

0.5 c.c. of mid-piece diluted	Un- heated	Heated at 55° C. for			Un- heated	Heated at 55° C. for				
		15 minutes	30 minutes	1 hour		15 minutes	30 minutes	1 hour	1½ hours	2 hours
1. 1 in 10										
2. 1 in 20										
3. 1 in 40										
4. 1 in 80										

To the various dilutions of mid-piece were added 0.5 c.c. of a 1 in 20 suspension of sheep corpuscles, and 0.5 c.c. of a 1 in 300 dilution of inactivated haemolytic serum. After preliminary incubation for half an hour were added 0.5 c.c. of a 1 in 10 dilution of unheated end-piece and 0.5 c.c. of saline solution. Haemolysis was then allowed to proceed for two hours.

A mixture was prepared of corpuscles 1 in 20, haemolysin 1 in 300, fresh mid-piece 1 in 20 and saline solution in equal parts. This mixture was incubated for half an hour and 2 c.c. of it were added to each dilution of end-piece. Haemolysis was allowed to proceed for two hours. Control tubes which showed that neither mid- nor end-piece was able, when acting alone, to produce haemolysis, were also set up.

It will be remembered that Muir and Browning (1909) published experiments in which it was found that guinea-pig complement would not pass through a Berkefeld filter. The filters used by us may have been more permeable than those used by Muir and Browning, but the experiments performed by us with whole serum were too few to enable us to reach a definite conclusion on this point. Our experiments did, however, show that whole guinea-pig serum could pass through the filter which we used, without appreciable loss in the value of the complement, while a salt solution preparation of the mid-piece was completely inactivated by passing through the same filter. The fact that the mid-piece was retained by the filter must probably be attributed to the size of the particles of the globulin suspension.

The state of the globulin in a salt solution suspension is probably quite different from its state in a saline dilution of normal serum, and some of the results obtained with the use of the mid-piece preparations are quite possibly due to the physical state of the globulin and not to any specific action of the mid-piece fraction of the complement.

*On the Relative Proportions of Mid-piece and End-piece
necessary for Haemolysis.*

Fränkel (1911) made several experiments with mid- and end-piece in varied proportions. He concluded that within a narrow range decrease of one fraction could be compensated by increase of the other. Occasionally, however, when rather small amounts of end-piece were used, haemolysis was inhibited by an increase of the amount of mid-piece in the mixture. Fränkel also noted that mid-piece could be diluted further than end-piece.

The inhibitory action of relative mid-piece excess is also recorded by Marks (1911), who concluded that it was possible that complement underwent physico-chemical alterations during the process of separation whereby a new proportion between mid- and end-piece became necessary for the attainment of the best results. Marks also stated that the inhibitory influence of mid-piece excess might be abolished by the use of preliminary persensitisation.

Table IV gives a typical result of an experiment in which various quantities of mid-piece were titrated with various quantities of end-piece. The haemolytic value of the whole guinea-pig serum from which the fractions were prepared is given in the right hand column. It will be seen that the mid-piece could be diluted to a greater extent than the

TABLE IV.

To show the proportions in which mid-piece and end-piece produce haemolysis.

0.5 c.c. of mid-piece diluted	1 in 10	1 in 20	1 in 40	1 in 80	1 in 160	1 in 320	1 in 640	+0.5 c.c. saline solution (mid-piece controls)	0.5 c.c. of whole guinea-pig serum diluted
(1) 1 in 10									1 in 10
(2) 1 in 20									1 in 20
(3) 1 in 40									1 in 40
(4) 1 in 80									1 in 80
(5) 1 in 160									1 in 160
(6) 1 in 320									1 in 320
(7) 1 in 640									1 in 640
0.5 c.c. of saline solution (end-piece controls) ..									
(8)									

All the tubes of the experiment proper contained 0.5 c.c. of a dilution of mid-piece, 0.5 c.c. of a dilution of end-piece, 0.5 c.c. of a 1 in 20 suspension of sheep corpuscles, 0.5 c.c. of a 1 in 400 dilution of a haemolytic serum (rabbit v. sheep cells) and 0.5 c.c. of normal saline solution. The corpuscles, haemolytic serum and mid-piece were mixed and incubated for half an hour at 37° C. End-piece was then added and after a further period of incubation for two hours at 37° C., the results were read and recorded.

The mid-piece and end-piece control tubes contained an extra 0.5 c.c. of saline solution. On the right hand side of the Table are given the corresponding dilutions of the whole guinea-pig serum from which the fractions were prepared.

end-piece. This result was often more marked than in the experiment here recorded. It was, as a rule, found that the end-piece could be diluted to about the same extent as the whole complement, but that the mid-piece could be diluted considerably further provided that a sufficient quantity of end-piece was added. The action of the smaller quantities of mid-piece was, as a rule, improved by increasing the quantity of end-piece in the mixture. Great relative excess of end-piece seemed in some of the experiments to exercise a slight inhibitory action. The inhibitory effect of great relative excess of end-piece was not a constant phenomenon in these experiments and was never well marked. On the other hand relative mid-piece excess had a very pronounced inhibitory action. This inhibitory action was much more marked when mid-piece and end-piece were added simultaneously. When mid-piece, corpuscles and haemolysin were submitted to a period of incubation before the addition of end-piece, the inhibitory action was less marked.

We have made numerous experiments with different samples of mid- and end-piece and with results similar to those recorded in Table IV.

We do not agree with Fränkel's conclusion that decrease of one fraction can be compensated by increase of the other. It is quite true that the action of relatively small amounts of mid-piece can be increased by increasing the amount of end-piece. If, however, a relatively small amount of end-piece is used, haemolysis is inhibited and not favoured by increasing the quantity of mid-piece in the mixture.

Experiments dealing with the inhibitory action of the mid-piece fraction will be described in a subsequent paragraph.

Effect of adding fresh Mid-piece Preparation to whole Complement.

The effect of adding the separated fractions to whole complement was investigated by Fränkel who found that both fresh and old preparations of mid-piece as a rule had an inhibitory action on the whole complement. Occasionally, however, haemolysis was improved by the addition of mid-piece. The addition of end-piece either fresh or old always improved the action of complement.

Hecker (1907) also stated that the action of either whole complement or of an active mixture of mid- and end-piece could be inhibited by the addition of the mid-piece.

The following table shows the results obtained by adding mid-piece in various amounts to various amounts of whole complement.

TABLE V.
Effect of adding fresh mid-piece solution to whole complement

(Simultaneous mixture of whole complement and mid-piece.)		+0.5 c.c. dilutions of whole guinea-pig serum						(Previous mixture of mid-piece, corpuscles and haemolysin and subsequent addition of whole complement.)					
0.5 c.c. dilution of mid-piece solution		1 in 10	1 in 20	1 in 40	1 in 80	1 in 160		1 in 10	1 in 20	1 in 40	1 in 80	1 in 160	
1. 1 in 10													
2. 1 in 20													
3. 1 in 40													
4. 1 in 80													
5. 1 in 160													
6. 1 in 320													
7. 0.5 c.c. saline solution													

Each tube (excepting the tubes in row 7) contained 0.5 c.c. of corpuscles (1 in 20), 0.5 c.c. of haemolytic serum (1 in 400), 0.5 c.c. of diluted whole guinea-pig serum, 0.5 c.c. of mid-piece dilution and 0.5 c.c. of saline solution. In one series all the ingredients were mixed at the same time, in the second series the corpuscles, haemolysin and mid-piece were mixed and incubated for $\frac{3}{4}$ hour. The complement was then added. Row 7 shows the haemolytic value of the whole complement without the addition of mid-piece. In the case of each series haemolysis was allowed to proceed for two hours at 37° C.

It will be seen that the smaller quantities of the mid-piece preparation greatly increased the haemolytic action of the smaller, and by themselves insufficient, quantities of whole complement. The larger quantities of the mid-piece had a pronounced inhibitory action on the smaller quantities of whole complement.

When the corpuscles, haemolysin, whole complement, and mid-piece were simultaneously mixed together, this inhibitory action was very marked. When the corpuscles, haemolysin and mid-piece were allowed a preliminary period of incubation before the addition of the whole complement this inhibitory action was diminished, while the supplementary action of the smaller amounts of mid-piece was enhanced.

A suspension of euglobulin if present in sufficient amount acts by interfering with the action of the end-piece fraction of the complement whether the end-piece is added as a preparation of pure end-piece or in a dilution of whole complement. These results are in accord with the results obtained in the experiments made with a view of determining the optimal proportions of the two fractions. The inhibitory action of relative mid-piece excess, was more evident in those experiments in which there was a simultaneous mixture of mid-piece, end-piece, corpuscles and haemolysin.

The changes which occur, on keeping, in the separated fractions of the complement.

If freshly prepared mid-piece solution is added to freshly prepared end-piece solution the mixture can be used in the same way as a corresponding dilution of the whole complement. That is to say if it is added to a suitable mixture of corpuscles and inactivated haemolytic serum, lysis of the corpuscles rapidly takes place.

If an interval is allowed to elapse after the separation of the two fractions a change is found to have taken place, in that very little haemolysis follows the addition of a mixture of the two fractions to sensitised corpuscles. The degree of lysis produced under these conditions is very slight and only becomes apparent after a considerable latent period. If, however, corpuscles, haemolytic serum and mid-piece solution be mixed and incubated for about fifteen minutes and if end-piece is then added to the mixture lysis is rapid and complete.

Brand (1907) who was the first to note the occurrence of this change explained the results by a further elaboration of Ehrlich's theory. Brand gave the name mid-piece (Mittelstück) to that fraction of the

complement which is contained in the globulin solution on the assumption that it fitted directly on to the complementophile group of the amboceptor. The second fraction of the complement, which remains in the supernatant fluid, was called the end-piece (Endstück) on the assumption that it completed the chain by becoming attached to the free end of the mid-piece.

The various components of the haemolytic reaction were supposed to form a chain linked in the following order: corpuscle, amboceptor, mid-piece, end-piece.

Hecker (1907) explained the change which takes place in the separated fractions by assuming an increased avidity of the mid-piece for the end-piece whereby it effects a union with the end-piece, but fails to couple up with the amboceptor.

Experiments intended to illustrate and explain the changes which occur, on keeping, in the fractions of the complement.

The first question to answer is whether the change occurs in the mid-piece or in the end-piece. To settle this point the following experiments were carried out:

(1) Two mid-piece preparations (see Table VI) were selected. The one of these was quite fresh and had been prepared on the day of the experiment, the other had been kept in the cold room for 13 days. These two mid-piece preparations were tested with six end-piece preparations, one of which had been prepared on the day of the experiment, while the others were 3 (two preparations), 4, 11 and 13 days old respectively. That is to say the two preparations of mid-piece were each tested with six different preparations of end-piece. The mid- and end-piece preparations were mixed in each tube and sheep corpuscles and haemolytic serum were then added. The result shows that complete haemolysis took place in all the tubes which contained the fresh mid-piece, while in the tubes which contained the old mid-piece no haemolysis took place. The mixture of 13-day old end-piece with fresh mid-piece produced haemolysis, while the 13-day old mid-piece produced no trace of haemolysis with end-piece prepared on the day of the experiment.

(2) Two end-piece preparations were selected. The one had been kept for 13 days, the other had been prepared on the day of the experiment. Each preparation of end-piece was tested with a preparation of fresh mid-piece and with five other preparations of mid-piece which had

TABLE VI.

Showing the results obtained by adding new mid-piece to old end-piece and by adding old mid-piece to new end-piece. Date of experiment Oct. 2nd.

0.5 c.c. of 1 in 10 dilutions of various end-piece solutions (date of preparation of end-piece)	+0.5 c.c. of a 1 in 10 dilution of mid-piece (prepared Oct. 2nd)	+0.5 c.c. of a 1 in 10 dilution of mid-piece (prepared Sept. 19th)	+0.5 c.c. saline solution
1. September 19th			
2. „ 21st			
3. „ 28th			
4. „ 29th (A)			
5. „ 29th (B)			
6. October 2nd			
7. 0.5 c.c. Saline Solution			

0.5 c.c. of 1 in 10 dilutions of various mid-piece solutions (date of preparation of mid-piece)	+0.5 c.c. of a 1 in 10 dilution of end-piece (prepared Oct. 2nd)	+0.5 c.c. of a 1 in 10 dilution of end-piece prepared Sept. 19th)	+0.5 c.c. saline solution
1. September 19th			
2. „ 21st			
3. „ 28th			
4. „ 29th (A)			
5. „ 29th (B)			
6. October 2nd			
7. 0.5 c.c. Saline Solution			

In this experiment the quantities of mid-piece and end-piece shown in the table were mixed together. To every tube was then added 0.5 c.c. of saline solution, 0.5 c.c. of a 1 in 20 suspension of sheep corpuscles and 0.5 c.c. of a 1 in 400 dilution of haemolytic serum (rabbit v. sheep). The results were read after incubation for two hours at 37° C.

been kept for various periods. The fresh mid-piece and two preparations of mid-piece which were three days old produced complete haemolysis both with the fresh and the old preparation of end-piece. The three older preparations of mid-piece produced haemolysis neither with the fresh nor with the old preparation of end-piece.

It should be added that if the 13-days old mid-piece was incubated for 20 minutes with corpuscles and haemolysin, the subsequent addition of either old or fresh end-piece produced complete haemolysis.

It is obvious that the inhibition of haemolysis which occurs when old mid- and end-piece are simultaneously added to corpuscles and haemolysin depends on some change which takes place in the mid-piece fraction. The end-piece fraction does not appear to undergo change.

It will be noticed that not only the fresh mid-piece but also the two preparations which were two days old produced haemolysis on simultaneous mixture with end-piece, corpuscles and haemolysin. It was found that great differences existed between the various samples of mid-piece in respect to the time which elapsed before they became unable to produce haemolysis when added simultaneously with the end-piece. Some samples underwent the change within a few hours of their preparation, others did not become changed for several days. The change which occurs is probably intimately connected with the changes which occur in the state of division of the globulin particles in suspension in normal saline solution.

In the preparation of the complement fractions the euglobulin of the serum is precipitated and carries down with it the mid-piece fraction of the complement. The euglobulin is then suspended in 0.85 per cent. saline and under favourable conditions the particles of the suspension are so fine that a perfectly translucent fluid is obtained. After an interval of from one to five days, the various samples varied greatly in this respect, the globulin particles tend to aggregate, the fluid becomes opalescent, then turbid, and finally the greater part of the euglobulin forms a deposit.

It is probable that after a certain stage of aggregation of particles has been reached the fluid inhibits haemolysis by acting on the end-piece fraction which becomes entangled in the small aggregations of globulin.

If, however, corpuscles, haemolytic serum and mid-piece be allowed a preliminary period of incubation the globulin is rapidly aggregated or precipitated on the surface of the corpuscles. It is then no longer present in suspension throughout the fluid and is no longer able to

exert an inhibitory influence on the end-piece. Experimental evidence that euglobulin is actually precipitated by a mixture of red cells and haemolysin or by a suitable mixture of serum and homologous antiserum has been published by one of us (Dean 1911).

*On the Mechanism of the Inhibitory Action of old
Mid-piece Preparations.*

The experiments recorded in Table VI show that some change occurs on keeping in the mid-piece preparation which tends to prevent haemolysis, if mid-piece and end-piece are simultaneously added to red cells and haemolytic serum.

The view was also put forward in the last paragraph that the process depends on the gradual aggregation of globulin particles which may be seen to occur in old suspensions of mid-piece in salt solution.

The following experiments (Table VII) show that (1) an old mid-piece preparation does not interfere with the combination of corpuscle with haemolytic serum and fresh mid-piece, (2) an old mid-piece has the power of directly inhibiting the action of fresh end-piece solution.

The first part of the table shows that old mid-piece solution does not interfere in the least with the union of fresh mid-piece antibody and red cell. The only result of adding the old mid-piece was to reinforce the action of the fresh mid-piece.

In the experiment recorded in the second part of the table the old mid-piece was allowed to act directly on the fresh end-piece solution. After half an hour the old mid-piece was found to have inhibited the end-piece markedly. It is quite clear that the old mid-piece acts directly on the end-piece.

This inhibitory action of the mid-piece on the end-piece is probably the direct result of the physical state of the euglobulin suspended in salt solution. It is most marked, as has been shown, in preparations which have been allowed to stand for some days, but it is demonstrable even in freshly prepared mid-piece solutions. It was always found, even with freshly prepared fractions, that better results were obtained by the preliminary incubation of red cells, haemolysin, and mid-piece and the subsequent addition of the end-piece, than when the mid-piece and end-piece were added simultaneously to the red cells and haemolysin.

TABLE VII.

Experiment to determine the mechanism of the inhibitory action of old mid-piece solution. Date of experiment Oct. 9th.

0.5 c.c. of various dilutions of a mid-piece solution prepared Oct. 9th	+0.5 c.c. saline solution	+0.5 c.c. of a 1 in 10 dilution of mid-piece solution prepared Oct. 2nd.
1. 1 in 10		
2. 1 in 20		
3. 1 in 40		
4. 1 in 80		
5. 1 in 160		
6. 1 in 320		

Duplicate sets of dilutions of fresh mid-piece were prepared. To each tube of one set was added 0.5 c.c. of saline solution, to each tube of the other set was added 0.5 c.c. of a 1 in 10 dilution of old mid-piece solution. To every tube was then added 0.5 c.c. of a 1 in 20 suspension of sheep corpuscles and 0.5 c.c. of a 1 in 400 dilution of haemolytic serum (rabbit v. sheep). After incubation for half an hour at 37° C., 0.5 c.c. of a 1 in 10 dilution of fresh (prepared Oct. 9th) end-piece solution was added to each tube. The amount of haemolysis was recorded after incubation for two hours at 37° C.

0.5 c.c. of various dilutions of an end-piece solution prepared Oct. 9th	+0.5 c.c. saline solution	+0.5 c.c. of a 1 in 10 dilution of mid-piece solution prepared Oct. 2nd
1. 1 in 10		
2. 1 in 20		
3. 1 in 40		
4. 1 in 80		
5. 1 in 160		
6. 1 in 320		

Duplicate sets of dilutions of fresh end-piece were prepared. To one set was added saline solution, to the other set the old mid-piece solution. The tubes were then incubated for half an hour at 37° C. There was also prepared a mixture of equal parts of sheep corpuscles (1 in 20), haemolytic serum (1 in 400), and fresh mid-piece solution (1 in 10). This mixture was incubated for half an hour and 1.5 c.c. of it was added to each tube shown in the table. The results were read after two hours incubation at 37° C.

On the Haemolytic value of the Fractions of Rabbit Complement as compared with the Fractions of Guinea-pig Complement.

A considerable number of experiments have been recorded in which the complement fractions obtained from the sera of different animals have been interchanged.

Liefmann and Cohn (1910) found that the mid-piece derived from sheep serum could be activated by guinea-pig end-piece.

Landsteiner (1911) found that rabbit mid-piece could be activated by guinea-pig end-piece.

Marks (1911) found that the mid-piece of the ox and of the sheep could be activated by guinea-pig end-piece.

Fränkel, who has made the most extensive observations on this point, succeeded in producing various degrees of haemolysis with the mid-piece fractions of man, ox, sheep, goat, pig, cat and pigeon by the addition of guinea-pig end-piece.

The experiments described hitherto have been concerned with the fractions separated from fresh guinea-pig serum. It was decided to try the action of the complement fractions of the rabbit in both the phagocytosis and the haemolytic experiments. Guinea-pig serum is, as is well known, a very active haemolytic complement, while rabbit serum has comparatively little power to complement inactivated haemolytic serum. Rabbit serum is on the other hand quite an efficient complement for phagocytosis. The following experiment (Table VIII) illustrates the haemolytic value of the fractions obtained from normal rabbit and guinea-pig serum. A portion of each serum was retained in order that the complement value of the whole serum might be estimated. The guinea-pig serum produced complete haemolysis in a dilution of 1 in 40, and three-quarters haemolysis in a dilution of 1 in 80. The rabbit serum even in a dilution of 2 in 5 failed to produce complete haemolysis. The table shows that the end-piece of the rabbit in the strongest dilution (1 in 10) which could be employed, failed to produce even a trace of haemolysis with either rabbit or guinea-pig mid-piece. The rabbit mid-piece, on the other hand, produced excellent lysis when

TABLE VIII.

Haemolytic value of complement fractions of rabbit and guinea-pig.

0.5 c.c. of dilutions of rabbit mid-piece	+0.5 c.c. rabbit end- piece diluted 1 in 10	+0.5 c.c. guinea-pig end- piece diluted 1 in 10	+0.5 c.c. saline solution	0.5 c.c. of dilutions of rabbit end-piece	+0.5 c.c. of a 1 in 10 dilution of guinea-pig mid-piece	+0.5 c.c. of a 1 in 10 dilution of rabbit mid-piece	+0.5 c.c. of saline solution
1. 1 in 10				1. 1 in 10			
2. 1 in 20				2. 1 in 20			
3. 1 in 40				3. 1 in 40			
4. 1 in 80				4. 1 in 80			
5. 1 in 160				5. 1 in 160			
6. 1 in 320				6. 1 in 320			
0.5 c.c. of dilu- tions of guinea- pig mid-piece				0.5 c.c. of dilu- tions of guinea- pig end-piece			
7. 1 in 10				7. 1 in 10			
8. 1 in 20				8. 1 in 20			
9. 1 in 40				9. 1 in 40			
10. 1 in 80				10. 1 in 80			
11. 1 in 160				11. 1 in 160			
12. 1 in 320				12. 1 in 320			

To the various dilutions of mid-piece were added 0.5 c.c. of sheep corpuscles (1 in 20) and 0.5 c.c. haemolytic serum 1 in 400. After incubation for half an hour the end-piece solution was added. The results were read after a second period of incubation lasting two hours.

A mixture in equal parts of sheep corpuscles (1 in 20), haemolytic serum (1 in 400) and mid-piece (1 in 10), was prepared and incubated for half an hour at 37° C. 1.5 c.c. of the mixture was then added to all tubes shown in the table. The results were read after incubation for two hours at 37° C.

TABLE VIII (*continued*).

To show the haemolytic value of the whole sera from which the complement fractions were prepared.

0.5 c.c. of dilutions of whole serum	rabbit serum	guinea-pig serum
2 in 5		
1 in 5		
1 in 10		
1 in 20		
1 in 40		
1 in 80		

supplemented by guinea-pig end-piece. The table indeed shows that this particular rabbit mid-piece was haemolytically more active than the guinea-pig mid-piece. Exactly the same result was obtained with another specimen of rabbit serum.

Similar results as regards haemolysis were obtained by splitting human serum and sheep's serum. The end-piece of the sheep and of man was found to have comparatively little haemolytic value, while the mid-piece preparations showed marked haemolytic power when "complemented" with guinea-pig end-piece.

It must be concluded that the well-known haemolytic virtues of guinea-pig serum depend on the lytic activity of its end-piece.

While the end-piece of the rabbit is of little value for haemolysis it acts excellently in phagocytosis (compare phagocytosis experiments in Part I). This result appears to indicate some difference between the phagocytic and haemolytic complement.

GENERAL CONCLUSIONS.

Phagocytosis System.

(1) Mid-piece as prepared by the CO₂ method is unable by itself to sensitise *B. typhosus* for phagocytosis.

(2) End-piece has a slight sensitising action by itself—an action which is facilitated probably by minute traces of mid-piece remaining after the CO₂ treatment.

(3) With mid-piece and end-piece acting in concert it has only rarely been possible to obtain values approaching those of the original complement.

(4) The addition of mid-piece to immune typhoid serum (tropin) either does not alter the value of the latter or considerably depresses it.

(5) When the mid-piece is “indifferent” or only slightly inhibitory to tropin, the final addition of end-piece gives good complementing.

(6) When the mid-piece is very depressant, the end-piece is no longer able to produce definite complementing.

(7) Heated mid-piece is not inhibitory in conjunction with tropin but its power of acting in the joint system of tropin, mid-piece and end-piece is destroyed.

(8) Mid-piece which has been passed through a Berkefeld filter is not inhibitory but is inactive in the joint system.

(9) End-piece is more resistant to heat than mid-piece and remains longer active on keeping.

(10) Removal of the mid-piece from a fresh immune typhoid serum, greatly increases the tropic action of the latter when used in high concentrations.

Haemolytic System.

(1) Preparations of the mid-piece fraction in 0.85 per cent. salt solution remain active for many weeks if kept at 0° C. Their activity is quickly destroyed by a temperature of 56° C. They are inactivated by passing through a Berkefeld filter.

(2) Preparations of the end-piece fraction in normal saline may be preserved at 0° C. for many months. They are destroyed at 56° C. but somewhat more slowly than the mid-piece fraction. Preparations of end-piece can be filtered through a Berkefeld but not through a gelatin filter.

(3) An end-piece preparation (in conjunction with a suitable dilution of the mid-piece) may be diluted to the same extent as whole serum. The mid-piece fraction (in conjunction with a suitable dilution of the end-piece) can be diluted to a greater extent than the whole serum.

(4) Relative excess of mid-piece inhibits haemolysis. By adopting the method of preliminary incubation of corpuscles, haemolysin and mid-piece (persensitisation) the inhibitory effect is greatly diminished, but not absolutely abolished.

(5) The addition of relatively small quantities of mid-piece to whole complement increases haemolysis; the addition of relatively large quantities of mid-piece inhibits haemolysis. If the persensitisation method be used the inhibitory effect of the large quantities is lessened while the favourable effect of the small quantities is increased.

(6) Preparations of mid-piece vary greatly in respect of the time which elapses before they become unable to produce haemolysis if simultaneously added to corpuscles, haemolysin and end-piece.

(7) This change which occurs in old mid-piece preparations is probably due to an aggregation in the particles of the globulin in suspension. The state of division of the particles of globulin is probably the cause of the inhibitory action of mid-piece preparations.

(8) The mid-piece preparations of the serum of man, the rabbit, and the sheep can be activated with guinea-pig end-piece.

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THE SPREAD OF PLAGUE.

*Being the Introduction to a Discussion
in the Section of Tropical Medicine at the Annual Meeting of the
British Medical Association, Birmingham, 1911.*

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in India.

In opening the discussion on this subject I propose to confine my remarks to the knowledge gained from the study of the present pandemic, that is, since plague escaped from Southern China and infected Hong Kong in 1894. I must perforce be concise. Time will not permit me to marshal all the evidence for the support of my statements; I shall, however, append sufficient references to enable any one to ascertain their foundations.*

In the study of this most recent pandemic, epidemiologists have had an advantage over their predecessors, owing to the discovery of the plague bacillus by Kitasato (1894) and Yersin (1894), shortly after the appearance of the disease at Hong Kong. How far knowledge of the disease as manifested in recent years may be capable of explaining the epidemics in Europe during the Middle Ages is a question I shall return to later.

Immediately before 1894¹ plague was known to exist, and to be more or less endemic, in Assyria, in Arabia bordering on the Red Sea opposite Suakin; Mesopotamia, in the basin of the Tigris and Euphrates; North-East Persia, around the southern coasts of the Caspian; Uganda, on the south-west shores of the Nyanza; Gharwal and Kumaon, on the lower slopes of the Himalayas, bordering on Thibet; on the eastern side of Lake Baikal in Siberia, near the Mongolian and Manchurian frontiers; at Solenko in Manchuria; Yunnan, a hilly district in South China just north of Tonkin; Pakhoi, a Chinese port in the Gulf of Tonkin, and various towns between this last and Yunnan.

* The dates in brackets after authors' names in the text refer to the year the observations were published. The full titles and references to the journals, etc., will be found on consulting the bibliography at the end, where this date appears against the author's name.

¹ J. Netten Radcliffe, *The Progress of Leprosy in Persia*, 4th ed., Rep. L.G.B. Supplement, 1881; F. G. Clemow, *The Endemic Centres of Plague*, *Journ. Trop. Med.*, vol. 2, 1899-1900, p. 200.

The map attached shows roughly the situation of these foci, but before leaving them I will point out that in five out of the eight the disease was *known* to be associated with an epizootic of plague-like disease amongst rats or tarbagans (*Arctomys bobax*). The three areas where no such association has been recorded are in Arabia, Mesopotamia, and Persia, districts whence accurate information is with difficulty forthcoming.

The spread of plague throughout the world during the last fifteen years I can only indicate in the briefest manner.² Early in 1894 it was raging at Canton, and shortly after appeared in Hong Kong. These ports, especially the latter, have extensive shipping connexions with the rest of the civilized world. During 1895 plague appeared in a number of neighbouring Chinese ports, and next year (1896) extended to Bombay and the island of Formosa. In India it has in the last fifteen years spread over the country and been responsible for upwards of 7,000,000 deaths. In 1898 an extension to Jeddah, Madagascar, and Mauritius occurred, and in the following year the disease became disseminated over a wide area of the globe, appearing in Japan, Straits Settlements, Persian Gulf, further Red Sea ports, Alexandria, Mozambique, French Ivory Coast, Laureço Marquez, Honolulu and New Caledonia, Oporto, and South America. In 1900 plague was present in every quarter of the world—Europe, Asia, Africa, North and South America, and Australia—and the further history of the pandemic is largely a chronicle of intracontinental expansion. The situations where epidemics have occurred and the dates of the more important extensions may be gleaned from a glance at the accompanying maps, the first of which shows the endemic foci in 1893, and the second the positions where plague has existed up to 1909. The dates of its first presence in epidemic form are placed against the more important expansions. Plague has followed the trade routes, and long stretches of ocean have been no effective bar to its progress.

From a number of the towns marked as having experienced an epidemic no human cases have been recorded for the last year or two. Many of them, however, still have more or less rat plague every season. Indeed, I imagine that there are now few large ports in which plague does not smoulder amongst the rat inhabitants.

The Infectivity of Plague.

The universal experience in India has been that bubonic plague, whether it has become septicaemic or not, is for practical purposes not infective unless it develops a secondary pneumonia.³

² Bruce Low, *The Progress and Diffusion of Bubonic Plague from 1879 to 1898*, Supp. Twenty-eighth Ann. Rep. L.G.B., 1899; *Reports and Papers on Bubonic Plague, 1898-1901*, L.G.B. Report, 1902; *Progress and Diffusion of Bubonic Plague*, Supplement to Annual Report L.G.B. for each year. Surgeon-General Eager, *The Present Pandemic of Plague*, Pub. Health and Marine Hosp. Service, U.S.A., 1908.

³ Report of Indian Plague Commission, 1898-99 (1901), vol. v, pp. 88-90.

The same conclusion was reached by Ashburton Thompson (1900-7, 1906) in Sydney, by Blackmore (1902) in Port Elizabeth, by Hill (1904) in Natal, Artola (1903) in Callao, and Rabinowitsch and Kempner (1903) in Odessa. The relative frequency of single and multiple cases in individual houses was studied by Thompson in Sydney in 1900, by Pearse (1905) in Calcutta 1904, and by the Commission for the Investigation of Plague in India in 1905-7. The results showed that multiple cases were the exception and not much more frequent than could be accounted for statistically by the operation of chance.⁴

Pneumonic plague, on the other hand, is eminently infectious, and gives rise to further pneumonic cases. All persons infected through the lungs may not, however, exhibit pneumonic symptoms in a striking degree. In some the bacilli rapidly reach the blood stream and are from the first primarily septicaemic. Although numerous small outbreaks of purely pneumonic plague have arisen in China, India, Australia, Egypt, and elsewhere, the present pandemic has been characterized by its general restriction to the bubonic type, and the only large outbreak of pneumonic plague has been the one last winter in Manchuria.

The Origin and Spread of Pneumonic Plague.

Pneumonic plague may arise by the inhalation of bacilli into the lungs, where they rapidly multiply and early gain access to the blood stream, or bacilli which have gained entrance through any other channel and have become generalized may subsequently establish themselves in the lungs and occasion a secondary pneumonia. Some degree of secondary pneumonia is not uncommon in man and animals suffering from bubonic plague. A case of bubonic plague may therefore become a potential source of a pneumonic outbreak. The spread of the pneumonic form of the disease offers no difficulties, since it was shown by Childe (1897) that the sputum of these cases contains innumerable bacilli, and by Martini (1901) that plague pneumonia is readily produced in animals exposed to an atmosphere containing droplets of an emulsion of plague culture. Pneumonic plague is obviously spread by man-to-man infection, and is easily controlled by early isolation of patient and contacts. Moreover, the recent extension of plague throughout the world has not been due to this variety, so that it need detain us no further.

The Spread of Bubonic Plague.

The problem of the spread of the comparatively non-infective bubonic variety of plague has been much more difficult to solve. As the patient is not usually a source of danger to the community, the mechanism by which the disease spreads must be looked for elsewhere.

⁴ Reports, *Journal of Hygiene*, vol. vii, 1907, p. 877 et seq.

Rats and the Spread of Plague.

The association of rat plague and human plague forced itself upon the attention of epidemiologists and medical men in China from the outset—Rocher (1874), Lowry (1883). I have already referred to the fact that this association had for long been observed by the inhabitants of Gharwal, of Uganda, and of Yunnan, and that evacuation of villages on the first occurrence of rat mortality was a hygienic measure recommended by these primitive people.

The inhabitants of the province of Kwansi, in South-West China, had had experience of plague for twenty years before 1894, and the causal relationship of rat plague to the human disease was firmly believed in by them. They called the plague the "rat disease," and regarded the sick rats as the "messengers of the devil."

Simpson (1903) in 1902 addressed a series of categorical questions regarding their experience of the epidemiology of plague to forty practitioners in South-West China, and elicited much interesting information. Eight recognized its essential concomitancy, but had no evidence of its precedence; twenty-nine had observed the fact that rat mortality occurred prior to human cases, and three failed to answer this question. Still better opportunities for the careful observation of the part played by rats occurred in Hong Kong, and the same close association was observed by Lowson (1895), Yersin (1894), Clarke (1900), Pearse (1903), Simpson (1903), Hunter (1904).

In India the importance of the epizootic was recognized by Snow and Weir (1897), Hankin (1898), Simond (1898), and the German Commission (1899). As plague became scattered over India the same fact impressed the numerous sanitary officers engaged in combating the epidemic.

From experience of the outbreak in Formosa in 1897 Ogata (1897) came to the conclusion that plague was primarily a rat disease, as he noticed that these animals sickened and died before men were attacked. The same conclusion was drawn by Kitasato, Takaki, Shiga, and Moriya (1900), from their experience of plague in Japan (1899-1900).

The relationship between the epizootic and epidemic was particularly accurately brought out by Ashburton Thompson, and Tidswell in Australia in 1900. On account of the comparatively small size of the outbreak, and the fact that it involved an entirely European population, a detailed investigation of each case was accomplished. From these observations Thompson concluded:

(1) That plague in rats preceded the first case which occurred in man.

(2) The epizootic area was coextensive with the epidemic area.

(3) The epidemic was due to communication of infection from rats to man.

Further evidence of the dependence of the epidemic upon the epizootic has been derived with each successive outbreak, and with each outbreak in a new locality: South

Africa—Blackmore (1902), Hill (1904), Pakes (1905), Mitchell (1906); Mauritius—Vassal (1906); Egypt—Gotschlich (1900); Odessa—Rubinowitch and Kempner, Skschivan, Wernitz (1903); South American—Agote and Medina (1901); Uriarte (1900); Artola, Arce, and Lavereria (1903), and many others.

The evidence is so universal that it may be stated that during the last fifteen years no epidemic of bubonic plague has occurred in which preceding or concomitant rat plague has not been discovered on adequate investigation. Further, as human cases of bubonic plague are not infectious, it follows that under present conditions, as regards the virulence of the bacillus, rat plague is essential to the existence of epidemic bubonic plague.

The Time Relation between the Epizootic and the Epidemic.

The consensus of opinion amongst practitioners in Southern China that the epizootic preceded the epidemic has already been mentioned.

Ashburton Thompson clearly demonstrated this for the Sydney outbreak in 1900, and a considerable amount of evidence pointing in the same direction was obtained by Hunter (1904) from observations in Hong Kong. The question was examined in a very thorough way by the Commission for Investigation of Plague in India in Bombay, and also two villages in the Punjab, during 1905-6.⁵

In Bombay an average of 1,500 rats were examined weekly over a period of fifteen months, and the proportion plague-infected determined. For the purpose of instituting a comparison the percentage above or below the mean proportion for the year of plague-infected rats, and also the percentage above or below the mean for the year of plague cases are together plotted for each week in the diagram below (Fig. 3). From the figure it is seen that rat plague rises and falls a few weeks prior to the human epidemic. The same result was obtained from the data collected in the Punjab, and from villages in the neighbourhood of Bombay. The fact that the epizootic precedes the epidemic does not prove that man derives the disease from the rat. Both might be infected from the same source, but, owing to some unknown condition, the rats more readily acquire the disease. It is, however, consonant with the conclusion which has been arrived at on other grounds, namely, that although rat plague exists in epidemic form without the participation of man, the converse proposition does not hold.

How is Infection Conveyed from Rat to Man?

Two ways in which the plague bacillus may be transferred from a sick rat to a human being have suggested themselves:

⁵ Reports, *Journal of Hygiene*, vol. vii, pp. 724 to 798.

1. Through the excretions of the animal, which often contain the bacillus, being distributed broadcast and infecting the soil and foodstuffs.

2. Through the agency of some blood-sucking insect capable of transferring its attention from rat to man.

The adequacy of the first proposition depends firstly upon whether the excretions of rats contain plague bacilli in sufficient numbers to produce material infection of soil or foodstuffs, and secondly, whether, having been deposited on such material, the conditions are such as afford a reasonable chance of their being able to maintain their vitality and to multiply.

The Amount of Plague Bacilli in the Urine and Faeces of Rats Suffering from Plague, and their Adequacy to Occasion Infection.

Simpson (1903) records that of 554 infected rats which he examined, 91 (16 per cent.) had plague bacilli in the urine contained in their bladders. The Commission for Investigation of Plague in India⁶ quantitatively determined the number of plague bacilli in the urine of 21 plague-infected rats. In 6 cases the urine contained plague bacilli, but in small numbers compared with the quantity contained in the blood of the same animal, which often amounted to 100,000,000 per c.cm.; 11 out of 15 samples of the urine of patients dying of plague were found by the Commission⁷ to contain *Bacillus pestis*. With two exceptions, the smallest amount of such urine which would infect a guinea-pig by subcutaneous injection was 1 c.cm., and in no single instance did it occasion the death of an animal by rubbing it into the recently scarified skin.

The precise number of plague bacilli in faeces cannot be ascertained, as this material swarms with micro-organisms. If present in considerable numbers they may be discovered by rubbing the material on to a slightly scarified surface of a susceptible animal. Klein (1906) found that he could infect animals in this way with the sanguineous mucus obtained from the intestine of rats dying of plague. One is not justified in inferring from this that the faeces of plague rats are an important means of spread of the disease, either amongst rats or mankind. The Commission for Investigation of Plague in India made 139 experiments to ascertain whether infection could be readily produced by rubbing in the faeces of rats dead from plague. They produced infection with plague only three times.⁸

Further important evidence bearing on this question was obtained by the Commission,⁹ who kept hundreds of animals—rats and guinea-pigs—some inoculated with plague, the remainder healthy, living together, without cleaning them out. Fresh relays of inoculated animals were supplied to take the place of those dying of plague.

⁶ Report I, *Journal of Hygiene*, vol. vi, p. 519.

⁷ Reports, *Journal of Hygiene*, vol. viii, p. 221.

⁸ *Ibid.*, vol. vi, p. 522.

⁹ *Ibid.*, vol. vi, p. 450, and vol. x, p. 315.

Notwithstanding the fact that they took their food off the floors contaminated with the excreta of the sick animals, and in the case of the rats usually ate the carcasses of their dead companions, in no instance was an epidemic produced.

The facts at our disposal do not lend any support to the view that direct contact with the excreta of plague-infected rats is an important means by which the disease is spread.

The Chance of Survival of the Plague Bacillus if Deposited upon Soil and Floors under Natural Conditions.

Although, for the reasons advanced above, the excreta themselves might not constitute a serious danger, if the contained plague bacilli were to find themselves in circumstances favourable to their growth, they might multiply to such an extent as to render materials contaminated by them a source of infection. It must not be imagined, however, that because the plague bacillus can be readily grown on sterile media, the same is likely to occur in Nature. This organism is by no means hardy, and, although in a sheltered existence protected from all competitors it may endure even for years, it readily succumbs under the influence of drying, sunlight, and the unsuitable conditions occasioned by the presence of hardy saprophytes.

Viability of Bacillus pestis in Soils, etc.

Although frequently searched for, the plague bacillus has never been recovered from the floors of the huts in which several cases have occurred by any trustworthy observer. It will, however, survive for months in moist earth, if this is previously sterilized (Gladin, 1898; Marsh, 1901). If allowed to dry it succumbs rapidly, and can no longer be discovered after a few days (Rosenau, 1901, and Klein, 1904).

Unsterilized material from the floors of huts was grossly contaminated (soaked) with a broth culture of the bacillus by Mackie and Winter (Plague Reports¹⁰), but even then the organism could not be recovered after four days.

The Commission for Investigation of Plague in India made a number of experiments in which they sprinkled 100 c.cm. of broth culture of *Bacillus pestis* over three square feet of floor of cowdung or of cement. At intervals scrapings were taken and rubbed into the scarified skin of guinea-pigs. The cowdung floor retained its infectivity for forty-eight hours and the cement floor for twenty-four hours only. Needless to say, this was a very gross contamination, and the means taken to ascertain the continued presence of plague bacilli afforded much greater opportunities for infection than would likely occur in Nature.¹¹

¹⁰ Reports, *Journal of Hygiene*, vol. vi, p. 510.

¹¹ *Ibid.*, vol. viii, 1906, p. 509.

Chance of Survival of the Plague Bacillus if Deposited upon Foodstuffs.

The commonest stored food in India, and one to which rats have access, is grain. Hankin (1900) carried out a large number of experiments with different sorts of grain artificially and grossly infected with plague microbes. After five days he was unable even by subcutaneous injection to infect mice with the same. In rotten grain the bacillus died out in two to twenty-four hours. In the early days of plague in India grain came under suspicion, but there is no evidence that the grain and not the granary harboured the infection.

Hunter (1904) states that he discovered the plague bacillus in three samples of rice in Hong Kong. The account of his experiments is, however, not such as to enforce belief in this statement.

All evidence shows that small amounts of plague bacilli deposited on the surface of grain rapidly succumb to desiccation and have little chance of multiplying. Further, grains are not ingested raw, and as the *Bacillus pestis* is rapidly destroyed at 50° C., cooking annihilates them, so that one cannot imagine that the infection of grain by rats plays any material part in the spread of the disease.

That plague bacilli may multiply sufficiently to be a source of danger if accidentally sown upon some food material which is not destined to be subsequently cooked is undoubted, but the chance of a favourable culture medium and absence of competing organisms coinciding is remote. This means of spread could, however, hardly assume importance in India, where food already prepared for consumption is rarely stored.

Alimentary Infection.

The importance to be attached to the accidental contamination of foodstuffs by the excreta of rats depends upon the readiness with which infection by the alimentary canal occurs.

Feeding Experiments with Plague-infected Materials.

Wilm (1896) infected a monkey by allowing him to eat a piece of sugar-cane soaked in a culture of *Bacillus pestis*.

With rats negative results were obtained by Gibson (1898) and Simond (1898). Gibson prepared the ten rats which he used for the experiments by keeping them without food for twenty-four hours. He then fed them on small bundles of corn wrapped in pieces of gunny bags and then soaked in a broth cultivation of plague. The bundles of corn which were supplied to the rats that formed the subject of the feeding experiments were given to them in a moist condition. The supply of infected food was renewed as occasion required for about a fortnight. At the end of that time the rats, though each had consumed from 100 to 150 c.cm. of the original plague

culture, all remained well. Subsequently, with a view to testing their resistance, they each received hypodermically 1 c.cm. of the plague culture in question, with the result that they all succumbed to plague in thirty-six hours.

Hankin (1898-9) made similar experiments, usually with negative results. He is of opinion that rats contract plague from the intestinal canal only when large quantities of very virulent cultures are introduced into their food.

Simond's experiments go even further in this direction. In one experiment he caused a rat to drink a watery suspension of plague bacteria, which he made from a virulent agar culture of plague. In another experiment he fed a rat for three consecutive days on virulent agar cultures, one of these being administered daily. In certain further experiments he fed rats upon the organs and dead bodies of animals which had died of plague. In other experiments, again, rats were fed with pneumonic sputum and with food that was soiled with infected blood. In each and all of these experiments the rats remained healthy.

The Austrian and German Plague Commissions (1899) found that plague could be conveyed to rats by feeding them on plague cultures or the bodies of rats dead of plague.

The pathological changes found in the body after death pointed in most cases to the penetration of the bacillus through the mouth, and only in rare cases to the penetration through the intestinal wall. Out of 7 rats which were repeatedly fed in Bombay on plague-infected material 3 remained alive.

Kolle (1901) fed 60 white rats partly with bread soaked in cultures and partly with the organs of plague-infected rats, with 48 successful infections.

Simpson (1903) infected monkeys and rats by feeding on the blood or tissues of animals dying of the disease. He also is of opinion that he produced plague in calves, pigs, hens, turkeys, geese, ducks, and pigeons in the same way. The importance of this latter conclusion led to the repetition of similar experiments in Natal (Watkins-Pitchford, 1904), India (Bannerman and Kapadia, 1904), and the Azores (de Souza, Antonia, and others, 1910), but they failed to confirm Simpson's observations.

Kister and Schumacher (1905) did a large number of feeding experiments. They found that only 50 per cent. of rats fed developed the disease.

Berestneff (1906) fed white rats on the livers and spleens of septicaemic plague-infected guinea-pigs. There were twelve successful infections, nine rats dying between the third and sixth days.

Klein (1906), who had previously failed (with rare exceptions) to infect animals by feeding with fresh plague material, has recently succeeded in giving the disease to rats, mice, and guinea-pigs, by feeding them with gelatine cultures and organs of plague-infected animals (either alone or mixed with rice and wheat) which had been

dried, but not sufficiently to completely kill the contained bacilli.

The Commission for the Investigation of Plague in India (1905-6)¹² devoted particular attention to this question, with the following result:

It was found possible to infect rats with plague by feeding them with grossly contaminated material of a soft nature, 21.4 per cent. of wild Bombay rats being susceptible to this method of infection. In a series of similar experiments made with *M. rattus* in the Punjab 67.8 per cent. of the rats were susceptible, but in this series a much larger dose of infected material was given. Further, a large number of wild Bombay rats were infected by feeding them on the carcasses of their plague-infected comrades. No difference as regards the *post-mortem* appearances or the distribution of the primary bubo was found between rats infected in this latter way and rats infected by feeding on soft contaminated material. But, while the general pathological lesions found in all rats infected by feeding were in the main the same as those found in rats naturally infected, there were observed two most important differences. First, in naturally infected rats, while the primary bubo was as a rule in the neck (74 per cent.), the axillary and inguinal glands being also often affected (25 per cent.), in not one single instance, out of 6,000 *post-mortem* examinations, was a bubo found in connexion with the mesenteric glands in the abdomen. On the other hand, in the rats infected by feeding, the neck glands were involved in only 29 per cent. of the cases, the inguinal and axillary glands were practically never affected, while the mesenteric glands were the site of the primary bubo in no less than 72 per cent. of the infected animals. Secondly, in the case of naturally infected rats the stomach and intestines showed, as a rule, no marked pathological change, while in the case of rats infected by feeding, well-marked lesions were found in the intestines. It would appear justifiable to conclude, then, that in nature, infection of rats by feeding rarely or never takes place, and that rats do not become infected by eating the carcasses of their comrades. At least this method of infection can play no part in the epizootic spread of plague.

The conclusion to be drawn from these experiments is that animals susceptible to plague by inoculation may be infected by feeding, but that compared with introduction under the skin, an immense dose of highly virulent material is usually necessary to occasion infection. The few experiments which have been made with monkeys suggest that these animals are relatively more susceptible to take the disease by ingestion than rats or guinea-pigs.

The position of knowledge on the question of the importance of alimentary infection in the spread of plague may be summarized as follows:

1. Contamination of aliments may conceivably lead to the infection of human beings on occasion, but the chance of bacilli reaching foodstuffs destined for consumption uncooked, and in which they would multiply greatly, are slight.

2. The alimentary canal is not an easy method of infecting animals, large quantities of virulent bacilli being usually necessary.

3. There is absolutely no epidemiological evidence pointing to alimentary infection being anything but uncommon, and in about 75 per cent. of human cases, the situation of buboes indicates skin infection.

¹² Reports, *Journal of Hygiene*, 1907, vol. vii, p. 373.

The Transmission of Plague by Fleas.

We have now to consider the alternative proposition referred to on p. 1251 above—namely, that the infection is conveyed from rat to man by the agency of some blood-sucking insect. The possibility of insects playing this rôle has occurred to many investigators. Yersin (1894), Hankin (1898), and Nuttall (1897) showed that the dejecta of flies and ants fed on infected organs contained virulent plague bacilli. Ogata (1897) crushed fleas from rats which had died of plague and injected them into two mice, one of which died of plague after three days. He suggested, from epidemiological considerations, that plague was mostly conveyed by suctorial insects such as mosquitos and fleas.

Simond (1898) found organisms indistinguishable morphologically from the plague bacillus in the stomachs of fleas fed upon rats and mice dying of plague, and succeeded in infecting a mouse by injecting an extract of crushed fleas taken from a plague rat. From his observations on plague in India during the previous year Simond arrived at the idea that plague was transferred from rat to man by means of fleas, and he made some experiments to try to convey the disease from one animal to another by the agency of fleas. Having found that he was unable to transmit the disease by mere contact, when the animals were previously freed from any fleas they might have, he placed a rat dying from plague in a wide-mouthed bottle, and, *as it had only a few fleas upon it*, he added twenty fleas from a cat (species not identified). A young rat, enclosed in an iron box with a grating on one side, was lowered into the bottle. The rat which was affected with plague died after twenty-four hours, and its carcass was left in the bottle for thirty-six hours and then withdrawn. The other rat died of plague on the fifth day.

Tidswell (1900) found virulent plague bacilli in the stomachs of fleas taken from plague-infected rats, but failed successfully to repeat Simond's experiments on transmission from rat to rat by means of fleas. Zirolia (1902) found that *B. pestis* multiplied in the stomachs of fleas, and retained its original virulence for seven to eight days, during which time it was passed in the faeces.

Gauthier and Raybaud (1902, 1903) repeated Simond's experiments in Marseilles. In their experiments they employed a cage divided in the middle by two wire grills 2 cm. apart. The cage was placed in a glass jar. In one compartment was placed an inoculated white rat on which had been placed a dozen fleas captured upon rats from ships in the harbour. When the inoculated rat died, a healthy rat was placed in the second compartment, and after some hours had elapsed, during which the fleas transferred themselves from the dead to the living animal, the cadaver was removed. In this way Gauthier and Raybaud succeeded five times in conveying the infection from one rat to another; the number of negative experiments is not stated. An examination of the fleas found

upon the septicaemic animals showed the presence of *B. pestis*. They found, as was stated by Simond, that rats did not contract plague from one another by mere contact in the absence of fleas. The fleas used in their early experiments were not identified.

Tidswell (1903) made further attempts to convey plague from rat to rat by the agency of fleas, but was unable to do this.

Verjbitski (1904) carried out an extensive series of experiments on the flea transmission of plague in St. Petersburg. He showed that plague bacilli could be recovered from the stomach contents of fleas six days after they had fed on plague rats, and that the faeces of such fleas contained *Bacillus pestis* for about the same time. He succeeded in infecting rats with fleas from diseased rats fifteen times in seventy-six experiments. In each experiment ten possibly infected fleas were allowed to feed on the rat.

Verjbitski also made experiments similar to those of Gauthier and Raybaud described above, and out of forty experiments, using ten fleas apiece, infection was conveyed in four.

Liston (1905) pointed out that the common flea infesting rats in India was not *Ceratophylus fasciatus* or *Typhlopsylla musculi*, as in Europe, but a non-pectinated flea possessing considerable resemblance to *Pulex irritans*. This was first identified by Rothschild as *X. cheopis*, and is identical with the flea (*P. pallidus*) found by Tidswell to comprise 80 per cent. of the flea population of rats in Sydney and Brisbane. Liston observed multiplication of the plague bacillus in the stomach of this flea. Although his experiments on transmission, which were made by allowing fleas to bite first an animal suffering from plague and subsequently a healthy animal, were not successful, he brought forward much interesting and valuable circumstantial evidence in favour of the view that plague is so epidemically spread. He found that *X. cheopis* takes readily to another host when rats are not available, as is shown by the following observation. In March, 1903, an outbreak of plague occurred in the guinea-pig cages at the Zoological Gardens, Bombay. On visiting the gardens Liston was surprised to find the guinea-pigs, especially those which were sick, infested with rat-fleas (*X. cheopis*). Guinea-pigs do not usually harbour fleas, but dead rats had been found in the neighbourhood of the guinea-pig cages, and Liston imagined that the fleas on the guinea-pigs were derived from this source. Acting on this idea, he subsequently made use of guinea-pigs as traps for rat-fleas, and in this way was able to discover their presence in plague-infected huts on several occasions. That *X. cheopis* will attack man was also demonstrated by Liston.

Kister and Schumacher (1905) repeated in Hamburg the experiments of Gauthier and Raybaud. Their method was similar to that of the Marseilles observers. They take no note of the species of fleas upon the rats used, and the

amount of infestation of their experimental animals was apparently slight. All their experiments were negative, and they concluded that flea-transmission is neither usual nor important.

When the Commission for the Investigation of Plague in India was appointed in 1905, a prominent item in the programme drawn up by the advisory committee was the investigation of the part, if any, played by fleas in the spread of plague from rat to rat and rat to man.

Experiments in Cages.

After several failures a command of the conditions necessary was attained, and it soon became a simple matter to transmit plague from animal to animal by means of rat-fleas.

In the first series of successful experiments a glass case containing two wire cages side by side, each standing in a tin tray which collected the urine, was used. Both trays were filled with sand, in order to provide dryness and shelter for the fleas. Each cage was furnished with a lid through which the rats were introduced, and food and water given to them. The whole apparatus was covered in with fine muslin to prevent the escape of the fleas. A rat placed in one of these wire cages could not come in contact with a rat in the other cage nor with its urine or faeces.

The method of experiment was as follows: A plague-infected rat and a number of rat-fleas were placed in one of the wire cages. After the rat died, a fresh healthy rat was put into the other cage, the corpse of the infected rat being left in for twenty-four hours longer.

Sixty-six experiments with English white rats and with Bombay wild rats were done in this way, with the result that thirty healthy rats contracted plague; that is to say, there were 45 per cent. of successful transmissions. In all cases a fairly abundant supply of fleas was present; these could pass freely between the cages and, except for the air, formed the only apparent means of transmission of the bacilli from rat to rat.

In order to exclude aerial infection a second series of experiments was carried out, in which fleas were taken from a rat which had died of plague and were placed on a fresh rat in a clean flea-proof cage of similar construction to that already described, but containing only one wire cage. Twenty-one (55 per cent.) successful transmissions out of thirty-eight experiments were obtained in this way, both English white rats and Bombay wild rats being again used. The possibility of the rat-flea carrying plague from one rat to another was therefore demonstrated directly.¹³

Experiments in Specially Constructed Brick Animal Houses from which Fleas could be Practically Excluded.

The following experiments, which had as their object the determination of the relative importance of the Indian

¹³ Reports, *Journal of Hygiene*, 1906, vol. vi, pp. 435-450.

rat-flea, *X. cheopis*, and of actual close contact in the absence of fleas, in the dissemination of plague from animal to animal, were carried out in a series of small cabins, which were built especially for this purpose.

In these animal houses plague-infected animals, rats or guinea-pigs, were kept in close contact with healthy animals. The animals ran about together in a confined space, and ate out of the same dishes.

The plague-infected animals were allowed to die, and the corpses were not removed until some time after death. In some instances the concentration of infection was very great, in one case 21 infected animals being at one time in contact with 25 healthy ones. Further, the infection was often kept up for a long period by the daily addition of freshly infected animals. It is also to be noted that as the godowns were never cleaned out, close contact includes contact with urine and faeces of infected animals and contact with, and eating of, food contaminated with faeces and urine of infected animals.

In the case of the experiments with rats,¹⁴ the healthy frequently ate the carcasses of the infected ones introduced. Sixty-six series of experiments were carried out. Each experiment involved about 40 to 70 animals; 31 were control experiments in which no fleas were introduced.

In all these control experiments not one of the healthy animals contracted plague, whereas in those cases where fleas were present, an epizootic occurred, varying in extent and rate of spread according to the number of fleas present.¹⁵

Infectivity of the Animal Houses after an Epizootic had occurred, due to Infected Rat-fleas.

Some interesting experiments¹⁶ were carried out in the animal houses that had been used for some of the previous experiments.

Guinea-pigs and monkeys were introduced into them for one night. Some of the animals were in cages placed on the floor, others suspended 1 ft. from the floor, or in cages covered by fine gauze muslin, or surrounded by a layer of sticky fly-paper, 6 in. wide—that is, the simplest precautions to exclude fleas.

Of 13 unprotected monkeys 6 died; of protected none. Of 24 unprotected guinea-pigs 18 died; of protected none.

The important fact that the animal houses were infective three weeks after the last animal had died of plague in it was also brought out. This corresponds with the maximum time plague bacilli persist in fleas.

Experiments in Plague Houses.

The Commission subsequently repeated similar experiments in plague houses in Bombay.¹⁷ The infectivity of

¹⁴ *Journal of Hygiene*, vol. x, pp. 315-335.

¹⁵ Reports, *Journal of Hygiene*, vol. vi, pp. 450-467; vol. vii, pp. 421-436; and vol. x, pp. 315-335.

¹⁶ Reports, *Journal of Hygiene*, vol. vi, p. 461.

¹⁷ *Journal of Hygiene*, vol. vi, pp. 467-482; and vol. vii, pp. 436-446.

142 houses where cases had occurred or rats had died was tested by placing a guinea-pig in it overnight. The guinea-pig was subsequently segregated, and in 31 cases it died of plague.

Ninety-two experiments were made by placing two animals—monkeys, guinea-pigs, or rats—one protected by gauze or sticky paper, the other not, in suspected houses. Fifteen unprotected animals died; none of the protected. On 12 occasions fleas were removed from a rat which was found under suspicious circumstances and had presumably died of plague, carried to the laboratory and placed upon a healthy guinea-pig. In 9 cases the guinea-pig contracted the disease.

On 96 occasions fleas were taken off guinea-pigs, which had spent the night in a suspected house and transferred to a healthy animal. In 26 of these the animal died of plague, although not infrequently the guinea-pig from which they were removed escaped.

From the brief and quite inadequate account I have given, you must realize that a fine attempt to solve this problem has been made. I am not aware that such exhaustive experiments have ever been made before in the history of epidemiology, and the conclusion that in a plague-infected house the infection is due to infected rat-fleas, and not to an infection of the soil or the air, seems to me abundantly justified.

The Fate of the Plague Bacillus in the Body of the Rat-flea and the Mechanism by means of which the Flea Infects a Healthy Animal.

The blood is sucked up from the wound made by the pricker. This structure is composed of three parts, the epipharynx and the two mandibles. The apposition of the three forms a fine tube, up which the blood is drawn and passed down the gullet into the stomach by successive waves of contraction from before backwards of the muscles actuating the chitinous pharynx.

The stomach is a pear-shaped organ occupying a considerable part of the abdomen of the insect. The general anatomy of a flea and the arrangement of the mouth parts may be gleaned from the diagrams Figs. 4 and 5.

In order to arrive at the number of plague bacilli likely to be imbibed by a flea from the blood of a rat dying of plague, the Commission for Investigation of Plague in India made measurements of the capacity of the flea's stomach¹⁸ and of the number of live bacilli contained in the blood of rats dying or recently dead of plague.¹⁹

The average capacity of a rat-flea's stomach was found to be 0.5 cubic millimetre, and the number of bacilli in the blood of a plague-infected rat before death anything up to 100,000,000 bacilli per cubic centimetre.

If, therefore, a rat-flea imbibed the blood of such a rat it would receive into its stomach 5,000 germs, and from

¹⁸ Reports, *Journal of Hygiene*, vol. vii, p. 395.

¹⁹ *Ibid.*, vol. vi, p. 519.

the blood of a rat containing 10,000 germs per cubic centimetre 5 bacilli. It was found that the blood of about two-thirds of plague-infected rats contained more than this number of germs, so that rat-fleas in sucking the blood of plague rats in Nature have ample opportunity of taking at least a few bacilli into their stomachs.

Evidence of Multiplication of Bacilli in the Stomach of the Flea.

Evidence of multiplication was found by Ogata and Liston. In the experiments of the Commission fleas were fed on plague-infected rats until the death of the latter animals. They were afterwards fed on healthy animals, a fresh animal being supplied each day, so that it was impossible for them to imbibe any more bacilli. Each day a number of the fleas was dissected and the stomach contents examined as to the presence or absence of plague bacilli. In 5 to 30 per cent., according to the time of year, abundant germs were found up to the sixth day, and in one instance on the twentieth day.²⁰ The bacilli were often present in immense number, far more numerous than ever seen in blood, and massed together as in a culture. We have good evidence in this observation that multiplication of plague bacilli may take place in the flea's stomach. If there had been no multiplication, the bacilli in the blood originally imbibed would soon have been diluted and washed out by the new blood taken in at each feed on the healthy animals.

The Approximate Proportion of Fleas in the Stomachs of which Multiplication of Bacilli takes place.

A number of fleas removed from plague-infected rats were dissected, and the contents of the stomach examined by the Commission. The proportion containing organisms indistinguishable from plague bacilli was found to vary with the season of the year, being six times greater in the epidemic season than in the non-epidemic season. During the season of the year when the epidemic occurs the proportion of infected fleas for the first four days after removal from the plague rat was 43 per cent., on the sixth day there were 15 per cent., on the eighth day 16 per cent., and on the twelfth day 9 per cent. On one occasion the stomach contents of a twentieth-day flea were found full of plague bacilli. In the non-epidemic season only 5.2 per cent. were infected during the first six days.

The Duration of Infectivity of Rat Fleas taken from Plague Rats.

Fleas which had imbibed blood containing plague bacilli were fed on healthy guinea-pigs, a fresh animal being supplied each day until all the fleas had died. After being in contact with the fleas for twenty-four hours each guinea-pig was carefully cleared of fleas and segregated. By

²⁰ *Journal of Hygiene*, vol. vii, pp. 398-405.



Fig. 1.—Localities known to be plague-infected in 1893.



Fig. 2.—Localities plague-infected since 1894.

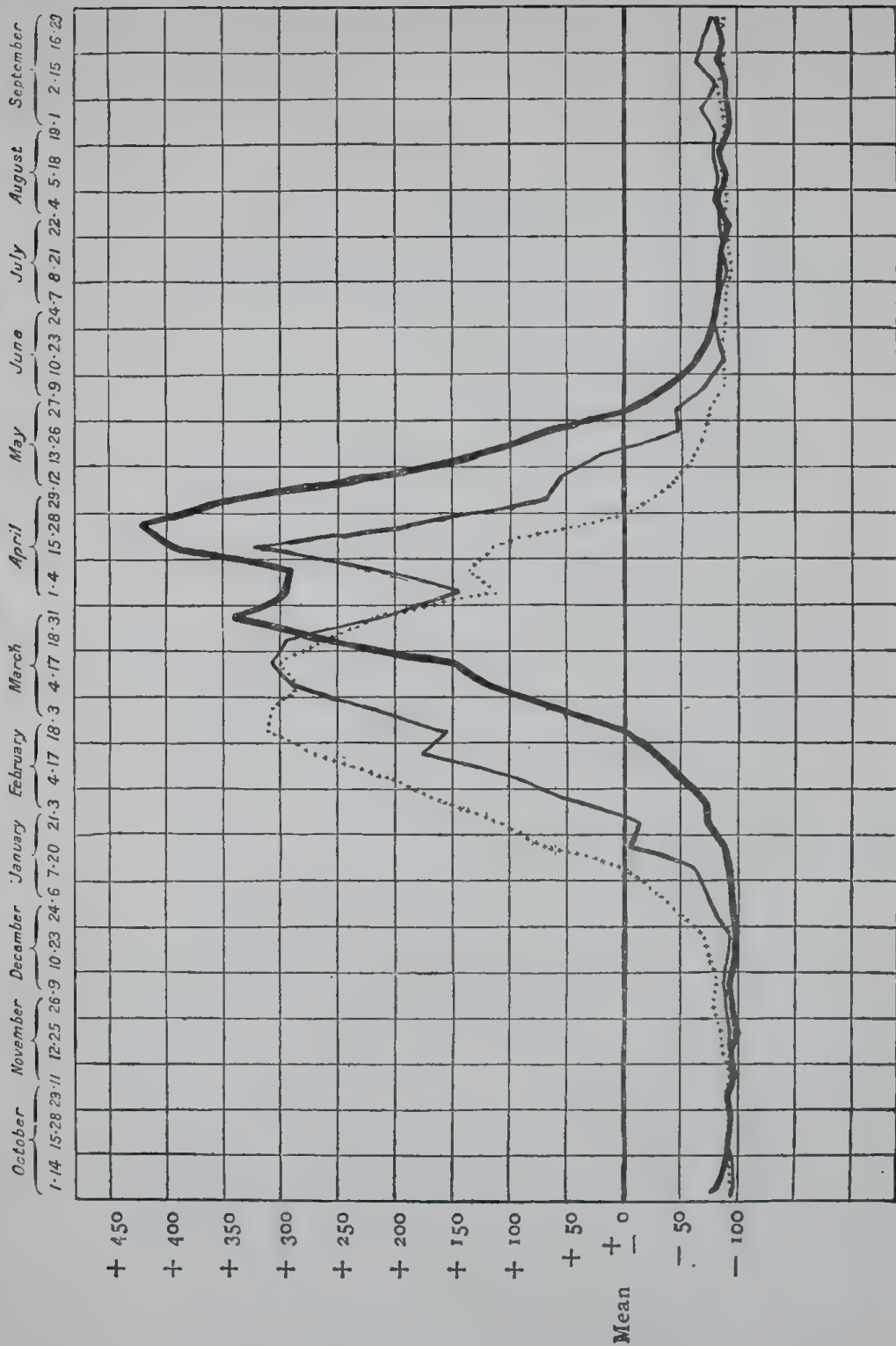


Fig. 3.—Chart showing the progress of the plague in rats and men in Bombay, 1905-6. Percentage above or below mean proportion of infected *M. decumanus*. — Percentage above or below mean proportion of infected *M. rattus*. — Mean of human deaths from plague.

this means it was ascertained that fleas might remain infective for fifteen days.

The Distribution of Plague Bacilli in the Body of the Flea.

The blood at the end of the digestive process in the stomach passes into the rectum of the flea as a thick, slimy, dark-red mass, and appears at the anus as minute, dark-red or black, tarry droplets. It is easy to demonstrate by dissection and examination that the rectal contents are often crowded with plague bacilli, and it has been repeatedly shown, both by microscopical examination and by cultural and animal tests, that the faeces may contain abundant and virulent plague bacilli.

On the other hand, a very large number of fleas, which had come off plague rats at intervals varying from a few hours to several days, have been dissected, and the various parts of the body examined for the presence of bacilli. On rare occasions a few bacilli have been seen in the gullet, but only in cases in which the flea has been killed immediately after feeding. As regards the other regions in the body, in not a single instance have any plague bacilli been observed outside the organs already mentioned. No infection of the body cavity has been seen, and, although particular attention was paid to the salivary glands, nothing at all resembling a plague bacillus has ever been detected in them. It would appear, then, that nowhere outside the stomach and the alimentary canal posterior to the stomach are plague bacilli to be found, and that they leave the body chiefly, if not entirely, along with the faeces.

How the Flea Transmits its Infection to the Healthy Animal.

It is evident that several methods of transmission are possible, such as (*a*) the animal eating the infected fleas; (*b*) the mechanical conveyance of the bacilli by the pricker; (*c*) the injection of the bacilli along with the saliva as the result of an infection of the salivary glands; (*d*) the regurgitation of the stomach contents or the retention of the infected blood about the mouth parts or in the pharynx, the bacilli being then injected with the saliva or carried down on the pricker or rubbed into the wounds made by the pricker; (*e*) the deposition of the infected faeces on the skin, the bacilli being subsequently rubbed in by scratching.

This last method is the only one which has been proved capable of bringing about infection. A well-fed flea deposits a considerable amount of faeces in a surprisingly short time. It has the habit of doing this either at the time of sucking or immediately afterwards, and it has been proved by both the Commission for the Investigation of Plague in India²¹ and Verjbitski (1904) that wounds

²¹ Reports of Plague Investigations, *Journal of Hygiene*, 1907, vol. vii, p. 418.

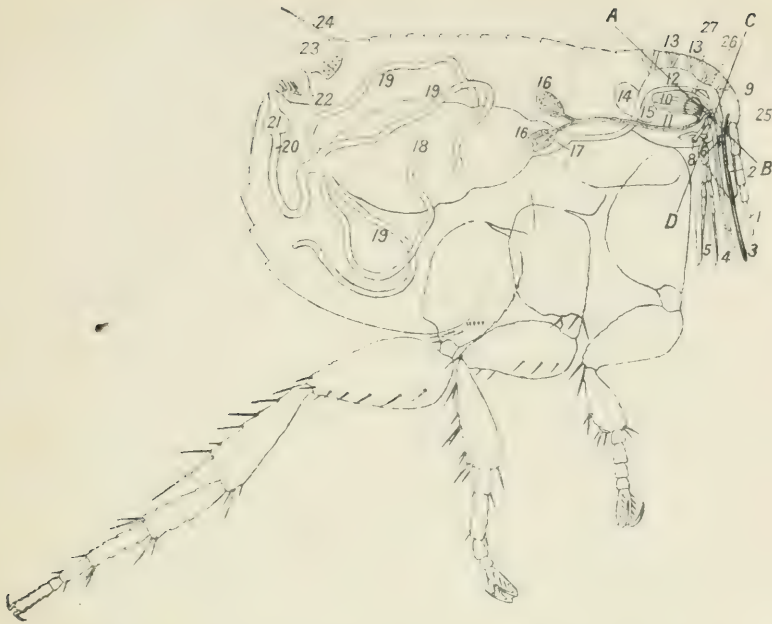


Fig. 4.—Diagrammatic mesial sectional view of *Xenopsylla cheopis*. 1, Maxillary palp; 2, maxilla; 3, epipharynx; 4, mandibles; 5, labium; 6, undivided portion of labium; 7, basal portion of labium; 8, basal portion of mandibles; 9, salivary pump; 10, hypopharynx; 11, salivary duct; 12, aspiratory pharynx; 13, muscles operating the aspiratory pharynx; 14, supraoesophageal ganglion; 15, suboesophageal ganglion; 16, salivary glands; 17, gizzard; 18, stomach; 19, Malpighian tubules; 20, rectum; 21, rectal glands; 22, claspers; 23, pygidium; 24, antepygidial bristle; 25, termination of dorsal contour of epipharynx; 26, mouth; 27, ligament.

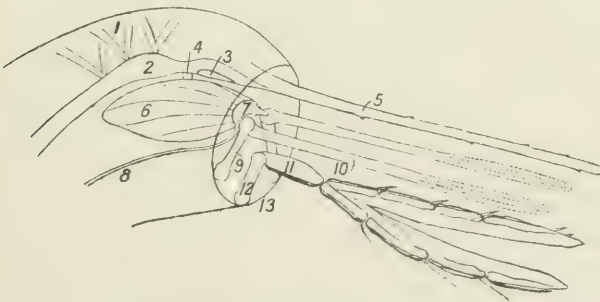


Fig. 5.—Diagram of the mouth-parts of the flea. 1, Muscles operating aspiratory pharynx; 2, aspiratory pharynx; 3, mouth; 4, ligament; 5, epipharynx; 6, hypopharynx and muscles operating salivary pump; 7, salivary pump; 8, salivary duct; 9, basal element of mandible; 10, mandible; 11, labium; 12, basal element of labium; 13, perioral ring.

made by the pricker afford a sufficient avenue for the entrance of bacilli when liquid containing them is gently applied to recent bites.

Whilst, however, experiments have shown that infection may be brought about in this way, the possibility of infection by contaminated mouth parts or by a regurgitation from the stomach as a preliminary to sucking blood cannot be excluded, though no evidence has yet been obtained in favour of either of these methods.

Fleas as the Agents of Transmission from Rat to Man.

In considering how far the results just detailed can be applied to man we enter at once upon less secure territory, because it is impossible to put conclusions to the test of experiment. The only measure of the correctness of such an interpretation is its adequacy to interpret all the known epidemiological facts concerning the spread of plague.

The justice of applying the results of animal experiments has been severely criticized in certain quarters, and particularly by Galli-Valerio (1907), on the ground that rat fleas do not bite man. It is true that, speaking generally, different animals harbour specific parasites; but the more we are learning on this subject the clearer it is becoming that such specificity is not so sharply defined as was imagined, and that a particular species of fleas, although commonly confined to a few hosts of allied species, may frequently be taken in considerable numbers off quite different animals. Moreover, it has become abundantly clear that a particular flea, although exhibiting a decided preference for one animal, will if hungry betake himself to animals of widely different species in the absence of its proper host.

Criticisms based on the supposition that rat-fleas do not bite man have arisen (1) from innocence of the variety and distribution of rat-fleas, and that they differ in different parts of the world;²² and (2) conclusions drawn from too meagre experimentation under an insufficient range of conditions.

The common, and in fact almost exclusive, flea found on rats in India is *X. cheopis*. This flea is a non-combed species, and not unlike the human flea in appearance. It appears to be the commonest flea inhabiting rats throughout the tropical and semitropical parts of the world, and to become less prevalent north and south of these regions, where it is supplanted by *Ceratophyllus fasciatus* or some other member of this genus. It is not uncommon on the rats in the ports of the warmer temperate regions having shipping connexion with the tropics, especially during summer and autumn. *X. cheopis* was recently found by Dr. Boycott (1911) to be abundant upon a particular colony of rats living in some warm cellars at Guy's Hospital.

²² The distribution of rat-fleas has recently been dealt with by Miss Chick and myself in the *Journal of Hygiene*, vol. ii, p. 129.

Numerous other fleas have been captured off rats, sometimes in considerable numbers, but these appear to be either occasional visitors or their presence in numbers is due to the particular mode of life of individual colonies of rats, leading to the sharing of parasites generally inhabiting some other animals, such as mice, fowls, cats, or field voles.

That *X. cheopis* readily feeds on man was observed by Tidswell (1903), Gauthier and Raybaud (1903), and Liston (1905). That under natural conditions they are attracted to man, the following extracts from the reports of the Commission for the Investigation of Plague in India show.²³ The extract also furnishes an idea of the degree of flea infestation in some of the habitations of the poorer natives.

April 13th.—113 fleas were caught on a man who entered one of the rooms. The species were as follows: *P. irritans*, 55; *X. cheopis*, 51; *P. felis*, 7.

April 19th.—76 fleas were caught on a man who entered one of the rooms for a short time. The species were as follows: *P. irritans*, 40; *X. cheopis*, 34; *P. felis*, 2.

April 20th.—80 fleas were caught on a man who entered one of the rooms. The species were as follows: *P. irritans*, 18; *X. cheopis*, 60; *P. felis*, 2.

The Commission for the Investigation of Plague in India kept *X. cheopis* alive for four weeks upon an exclusively human diet.

With regard to the readiness with which *Ceratophyllus fasciatus* attacks man considerable divergence of opinion has hitherto existed. According to Wagner (quoted by Tiraboschi 1904, p. 180), Tiraboschi (1904, p. 266), and Galli-Valerio (1907) this flea does not bite man. On the other hand, Gauthier and Raybaud (1903 and 1909) and McCoy and Mitzmain (1909) found that when hungry it fed on man with readiness.

Miss Chick and I (1911) have made some hundreds of experiments on this question, and are at a loss to understand the negative conclusion arrived at by Tiraboschi and Galli-Valerio. The fleas were starved for periods varying from twenty-four hours to fourteen days before testing their inclination to feed on man. The tubes were inverted upon the naked skin of the arm of the subject and the flea carefully watched for a period of two minutes. If it did not bite during this time it was regarded as negative for our experiments. Frequently the flea attached itself instantly or within a few seconds, and began to feed without delay; sometimes it took a little longer to get started, but usually, if it were going to bite at all, it did so before one minute had elapsed. As soon as the flea attached itself the tube was removed, and the behaviour of the insect was watched with a hand lens. The position assumed was generally characteristic. After a preliminary investigation of the surface with the tip of the labium and the maxillary palps, the insect was seen to press its head firmly on to the skin and raise its abdomen steeply into

²³ Reports, *Journal of Hygiene*, 1907, vol. vii, p. 475.

the air. Shortly afterwards blood was seen passing into the stomach in jets.

The results of 517 experiments with 364 fleas and 8 persons are given below in tabular form.

The proportion of fleas which bit varied from 36 per cent. to 77 per cent., giving a mean value for man of 59.6 per cent. The variation between different people is large enough to warrant the conclusion that the suggested idiosyncrasy is a reality.

In order to obtain a satisfactory control for these experiments, a similar set were undertaken, using the rabbit and the rat as subjects; the results are also given in the table. The experiments were in all details similar to the above, except for the expedients adopted for keeping the animal quiet during the observations.

The rabbit was rolled up in a cloth, and remained motionless on a table in a sleepy condition.

The rat was secured by a similar method. Two bandages were used, one for the neck and shoulders, and one for the thighs and legs, leaving a portion of the abdominal wall free. The area of skin upon which the fleas were tried was shaved. The rat was laid on its back, the head comfortably supported with a pillow of cotton-wool, and kept from rolling away by fastening the ends of the bandages to a board with drawing pins. The rat remained perfectly quiet, and there was no difficulty in carrying out the observations.

Under the conditions of these experiments *Ceratophyllus fasciatus* fed upon man as readily as upon a rat.

Although feeding readily enough, it is doubtful whether *Ceratophyllus fasciatus* can attack man as easily as *X. cheopis*. Compared with *X. cheopis* it is a poor jumper.

One hundred and seven experiments with *Ctenophthalmus agyrtes*, a flea common on *Mus decumanus* in this country, when living in fields, ricks, or barns, and 122 experiments with the mouse-flea *Ctenopsylla musculi* showed that under the conditions of the experiment the former would not bite man at all, and the latter only very occasionally.

This latter experiment is interesting because mice, although dying of plague, have not been found to be associated in the same way as rats with the origin of plague epidemics.

The Ability of Flea Transmission to Interpret the Epidemiological Facts and Conclusions Regarding Bubonic Plague.

The most prominent epidemiological fact concerning bubonic plague, namely, its essential dependence upon rat plague, has already been sufficiently emphasized. Many of the factors conducive to the incidence and spread of the disease, such as poverty, dirt, storage of grain in the bedroom, accumulation of refuse, and insanitary conditions generally, have been shown to be effective in so far as they lead to the support of a large rat population in close association with human beings. All these conditions also increase the population of rat fleas, and man's accessibility to them,

Table showing Results of Feeding Experiments with *Ceratophyllus fasciatus*.

	Subject.								Summary.	Rat.	Rabbit.
	C. J. M.	H. C.	A. M.	J. H. S.	H. Y.	G. F. P.	H. W. A.	S. R.			
Total number of experiments	161	118	39	33	52	51	40	23	517	101	32
Positive	106	65	30	12	36	25	19	15	308	59	23
Negative	55	53	9	21	16	26	21	8	209	42	9
Per cent. positive	65.8	55.1	76.9	36.4	69.2	49.0	47.5	65.2	59.6	58.4	71.9

but for the present I content myself with the statement that they are not inconsistent with the view of transmission by these insects.

I have also pointed out that the principal mechanism by means of which plague infection is conveyed from rat to man, must be such an one as will lead to the inoculation of the bacillus upon some superficial area of the body. This is also consistent with infection by fleas.

There remain one important epidemiological conclusion and one striking epidemiological fact concerning plague by which the validity of any hypothesis must be tested. These we will consider seriatim.

1. *Infection may be Conveyed to a Distance upon the Person, Clothing, or Effects of an Individual, even when the said Individual does not himself Contract the Disease.*

A number of instances have occurred during the last ten years in India in which the introduction into a distant village of the effects of a person dead of plague, or the visit of an individual who had worked or resided in a house where some of the inmates had suffered from the disease, has been followed after an interval of about a week by mortality amongst the rats and an epidemic. In many cases the movements of the inhabitants have been known, and the information has been of such a definite character as to leave no doubt in the minds of the sanitary officials concerned that the outbreak was causally connected with the introduction of the clothes or of the individual, *although the latter did not suffer from the disease.*

These observations are explicable on the assumption that the infection was transported in the bodies of fleas contained in the clothing or upon the persons of the individual, for at the earliest opportunity rat-fleas would betake themselves to the rats, and could thereby start an epizootic. That such transmission of rat-fleas does actually happen has been proved by the Commission for the Investigation of Plague in India, members of which frequently carried away rat-fleas upon their persons and clothing when visiting native quarters in the course of their scientific duties.

An experimental test of the adequacy of this method of transporting the infection was carried out by the Commission by introducing bundles of clothing from native houses where plague had occurred, and confining guinea-pigs with them in a special flea-proof cage. By the time they reached the laboratory the majority of the bundles of clothing contained no fleas, but a few fleas were so transported, and once in twenty trials the guinea-pig died of plague. A flea (this time a human flea) taken off another guinea-pig which remained healthy was found to contain numerous plague bacilli in its stomach.

The Seasonal Prevalence of Bubonic Plague.

Perhaps the most striking feature of epidemics of bubonic plague is the marked seasonal prevalence of the disease

amongst rats, and therefore amongst men. In places where it is endemic, the epizootic and epidemic start at or about the same time each year, grow, decline, and more or less disappear. As one passes away from the equator the plague season becomes later. In Bombay the height of the epidemic is in March, in Lahore April, in Jhelum May, in Rawal Pindi June, and further north July, August, and September. The epidemics of London in 1665, and of Marseilles in 1720, reached their maximum in September.

The effect of latitude in determining the season when plague flourishes is analogous to that upon the flowering and seeding of a plant, and indicates dependence on some biological factor. Within the tropics, where temperature variation is less, and the seasons are determined by the prevailing winds and rainfall, the epidemic season is also well defined, but varies in different localities.

It will be admitted that any interpretation of the spread of plague which affords an adequate explanation of the seasonal prevalence of the disease will receive thereby substantial support. The cause of this seasonal periodicity was sought for by Gotschlich (1903) in the seasonal breeding of rats. Gotschlich found that the greatest accession of young rats in Alexandria coincided with the epidemic.

The Commission for the Investigation of Plague in India made most extensive observations on the breeding season of rats in different parts of India.²⁴ In Bombay the proportion of females pregnant varied about 50 per cent. throughout the year, but the onset of the plague epidemic occurred in the season when breeding was at a minimum. In the Punjab breeding occurs more particularly in March and April, and again in September and October. These periods coincide with the onset of plague epidemics in this province. In Belgaum the rats breed all the year round, but more particularly during the early months of the year, which is a time when the plague epidemic is disappearing. In Poona the rate of breeding during the epidemic period was about half that of the non-epidemic period.

The only part of India where the season of greatest breeding of rats and the epidemic season for plague at all coincide is the North, but even in the Punjab there is no very definite breeding season. Although in the Punjab it may contribute to a small extent, a periodic accession of young susceptible rats cannot be the factor in determining seasonal prevalence throughout India.

The Coincidence of the Epidemic Season with the Period of Greatest Flea Prevalence.

In Bombay, Belgaum, Poona, and two Punjab villages the Commission undertook a census of rat fleas upon rats caught.²⁵ The fleas on 150,000 rats were recorded. In

²⁴ Reports, *Journal of Hygiene*, vol. vii, No. 6, Special Plague Number: vol. x, pp. 446-536.

²⁵ Reports, *Journal of Hygiene*, vol. viii, p. 266; vol. x, pp. 446 and 483.

each case the observations extended over more than one year.

The analysis of the figures showed a seasonal variation in the number of rat-fleas in all the localities. The average number per rat varied in Bombay between 3

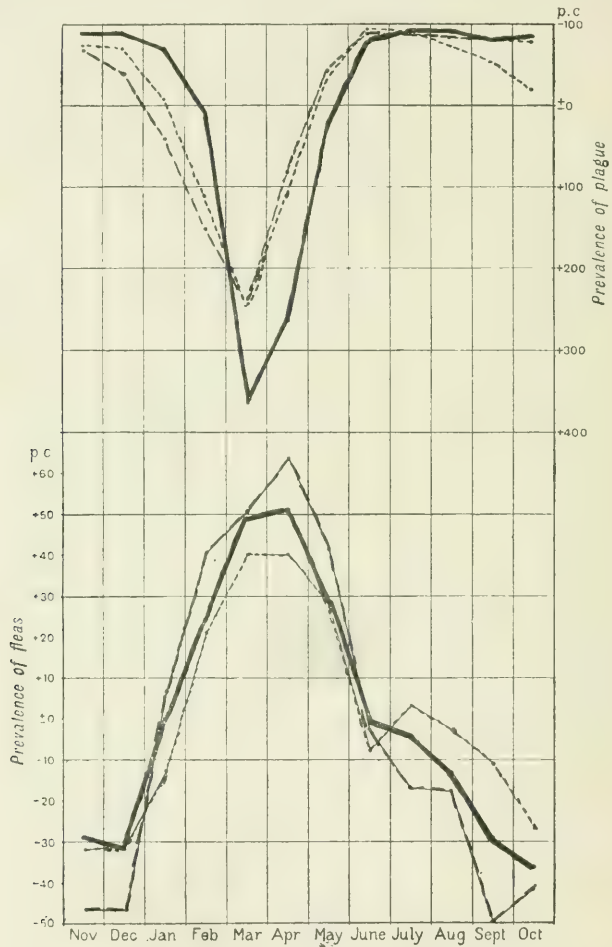


Fig. 6.—Prevalence of fleas in Bombay.

— Human plague. — Fleas on all rats.
 - - Plague in *M. decumanus*. - - Fleas on *M. decumanus*.
 . . . Plague in *M. rattus*. . . . Fleas on *M. rattus*.

and 7, in Poona between 2 and 11, in the Punjab 2 to 12, and in Belgaum between 1 and 17. The interesting point is that in all the localities examined plague is epidemic

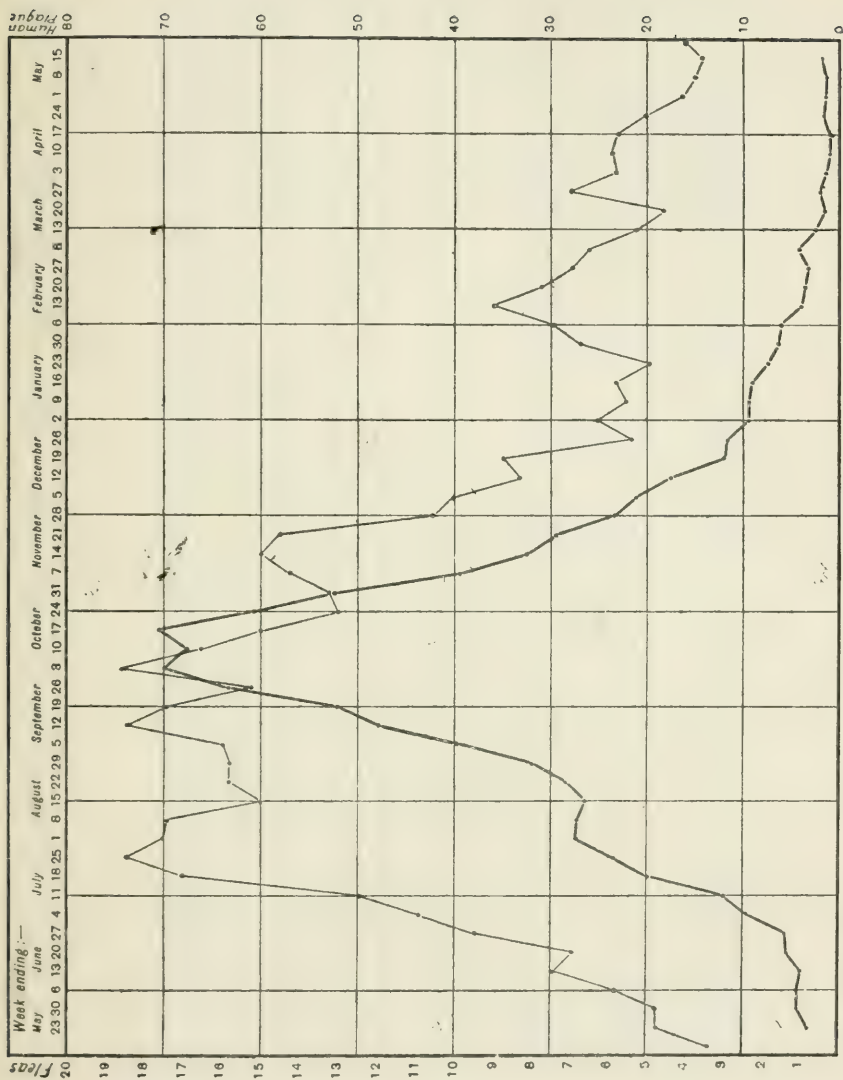


Fig. 7.—BELGAUM. ——— Average number of fleas per *Mus rattus*; weekly figures; observations made in 1908-1909.
 - - - - - Average number of deaths from plague, calculated for each week of the year, from May, 1897 to May, 1909 (twelve years), in Belgaum city.

when the average number of fleas is well above the mean, and the height of the epidemic corresponds fairly closely with the season of maximal flea prevalence. This coincidence is brought out in the attached charts (Figs. 6 and 7) showing the variations in fleas per rat and plague deaths each week for Bombay and Belgaum respectively.

A similar seasonal variation in the prevalence of *X. cheopis* and correspondence of the maximum of these fleas and the epidemic period has been observed by Kitasato (1909) in Japan, Tidswell (1910) in Sydney, and by Gauthier and Raybaud (1910-11) at Marseilles, and by Andrew (1911) in Tongschau in Northern China. The incidence of the epidemic period in the season of greatest flea prevalence under such different climatic conditions as obtain in Bombay, Poona, the Punjab, Belgaum, Sydney, Kobe, Tongshang, and Marseilles is in itself suggestive, and, in view of the results of animal experiments already referred to, one is justified in believing that the most favourable time for the epidemic is largely determined by the seasonal prevalence of rat-fleas.

2. *The Rat-flea Hypothesis Affords an Interpretation of the Fact that Epidemics Decline when the Mean Daily Temperature passes 85° F.*

From the comparison of the course of epidemics with the mean temperature for a number of localities in India and elsewhere the fact has emerged that plague does not maintain itself in epidemic form after the mean temperature has risen above 80° to 85° F.

It was not until the Commission for the Investigation of Plague in India found that in Bombay the proportion of successful experiments on flea transmission was greatest during the winter months that any light was thrown upon this rather curious fact. An analysis of their results gave 67 per cent. successful transmissions when the temperature was 73° to 78° F., against 14 per cent. successes when the temperature was 82° to 85° F. Transmission experiments were therefore carried out simultaneously at 70° and 85° F., both upon rats and guinea-pigs. The results were consistent with previous experience, the successful transmissions being two to three times as frequent at the lower temperature. The possibility that the virulence of the bacillus might undergo some variation in virulence if grown at one or other temperature was examined by the Commission and excluded. An explanation was subsequently found in the much greater rapidity with which plague bacilli disappear from the alimentary canal of the flea at the higher temperature.

The claims of flea-transmission to be the predominating mechanism of spread from rat to man may be briefly summarized as follows:

1. The experimental evidence that plague is easily transmitted from animal to animal by rat fleas.
2. That in presence of fleas, the epizootic, if started, varies as regards severity and rate of progress with the number of fleas present and the season of the year, whereas

all attempts to induce epizootics in the absence of fleas have failed.

3. That under natural conditions (experiments in plague houses, etc.) an animal can be protected from infection by any simple procedure which will exclude the visits of fleas.

4. The only discovered infection in plague houses resides in plague-infected fleas.

5. Rat fleas *X. cheopis* and *Ceratophyllus fasciatus* readily bite man.

6. The conclusions drawn from animal experiments, when applied to the problem of the spread of plague amongst human beings, afford a reasonable interpretation of every cardinal epidemiological fact.

The Part Taken by the Human Flea.

It is possible to transmit plague by means of *P. irritans*. Nevertheless, the direct transmission of the disease from man to man cannot, at the present time, be of frequent occurrence, or we should have evidence of direct infection instead of dependence upon the epizootic.

The reason why the human flea is ineffective is because in human cases the average degree of septicaemia before death is so much less than in rats that the chance of a flea imbibing even a single bacillus is small.

A variation of the plague bacillus in the direction of greater infectivity, with perhaps diminished toxicity leading to a higher degree of septicaemia in man, would permit of direct transmission by human fleas. Bubonic plague would then be independent of the rat, and spread directly from man to man. For several reasons it seems to me not improbable that this may have happened in the plagues of the Middle Ages.

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ATTEMPTS TO REPRODUCE THE TYPHOID-CARRIER STATE IN THE RABBIT.

(PRELIMINARY COMMUNICATION.)

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(*From the Lister Institute, London.*)

With Plate III.

SINCE the work of Blachstein and Welch (1891) who showed that rabbits inoculated intravenously with the typhoid bacillus might continue to harbour that organism in the gall-bladder for a prolonged period, much help in the elucidation of the typhoid-carrier state in man has come from the results of experimental investigation of this condition in the rabbit.

Among those who have contributed most materially to our knowledge of this subject one may cite the names of Forster and Kayser (1905), Doerr (1905) and Koch J. and Chiarolanza (1909), the latter of whom has shown that the infection of the gall-bladder following intravenous inoculation of *B. typhosus* in the rabbit most probably takes place directly through the blood vessels of the gall-bladder wall, although a secondary invasion by infected bile from the liver may certainly take place at a somewhat later period.

Histological examination of the gall-bladder and biliary tracts in such acute cases has revealed the presence of bacillary depôts of embolic origin at the extremities of the rugose papillary formations which give the mucosa the shaggy appearance so characteristic of typhoidal infection in the human gall-bladder.

In rabbits which have been killed at a later period after inoculation and found to be still harbouring the typhoid bacillus, very similar changes have been noted. Thus in one of Chiarolanza's experimental

rabbits which was sacrificed on the 14th day after inoculation, the gall-bladder mucosa showed inflammatory foci near the tips of the shaggy papillae, but bacilli could not always be demonstrated in them by staining methods, although a pure culture of the *B. typhosus* was obtained.

It is obvious that this method of experimentation offers a wide prospect of studying the carrier-state under laboratory conditions and particularly with a view to treatment. Already some preliminary experiments by Conradi (1910) and Hailer and Rimpau (1911) have appeared in which some success has been achieved in freeing the animal from typhoid bacilli by the intrarectal injection of Chloroform, Bromoform, Iodoform and other substances.

Most of these therapeutic attempts have been begun as a rule not later than the first fortnight after infection but a small series of animals was experimented upon by Conradi about the 25th day and in these cases recourse was had to preliminary laparotomy in order to determine whether the gall-bladders were really infected at this date.

The experiments which I propose to record in this short note were commenced in Oct. 1910 and the main object in view was to determine the frequency with which the chronic carrier condition occurs in the rabbit after intravenous inoculation and the length of its duration. It was also hoped that a clue to the infected condition of the gall-bladder might be obtained from the systematic bacteriological examination of the faeces. If such examination proved successful it was intended also to institute various therapeutic measures with a view to influencing the gall-bladder lesions.

Author's Experiments.

Between the dates Oct. 17, 1910, and Oct. 29, 1910, twelve rabbits with an average weight of 2.52 kilos received a single intravenous inoculation of 4 c.c. of a 24-hour broth culture of *B. typhosus*. In six of the animals a strain isolated from an intestinal carrier was employed, and in the other six a strain from a urinary carrier.

Two animals died within 12 hours from the effects of the injection.

The systematic examination of the faeces of the remaining ten rabbits was begun about ten days after inoculation. Before plating on MacConkey's medium the faeces were incubated for 24 hours at 37° C. in 0.5 % malachite-green broth.

The agglutinin-content of the rabbits' sera was also estimated at intervals, the homologous organism being employed.

Results in the individual rabbits.

Rabbit I. Wt. 2.50 kilos. Inoculated on Oct. 17, 1910.

Agglutinins: 31 Oct., 1 in 2000 + + +; 9 Nov., 1 in 2000 + + +; 15 Nov., 1 in 2000 +; 27 Nov., 1 in 2000 +; 13 Jan. 1911, 1 in 2000 + +; 21 Feb., 1 in 2000 +.

Faeces: Exams. on 1 Nov., 9 Nov., 17 Nov., 23 Nov., 1 Dec., 12 Jan., 4 Feb., 21 Feb., 1 Mar., 3 Mar. All negative.

Rabbit IV. Wt. 1.85 kilos. Inoc. Oct. 18, 1910.

Agglutinins: 31 Oct., 1 in 2000 + + +; 9 Nov., 1 in 2000 + + +; 15 Nov., 1 in 2000 + +; 29 Nov., 1 in 2000 +; 13 Jan., 1 in 2000 + +; 21 Feb., 1 in 2000 +.

Faeces: Exams. on 1 Nov., 9 Nov., 17 Nov., 23 Nov., 1 Dec., 12 Jan., 4 Feb., 21 Feb. All negative.

Rabbit V. Wt. 3.50 kilos. Inoc. 21 Oct. 1910.

Agglutinins: 31 Oct., 1 in 2000 + + +; 9 Nov., 1 in 2000 + + +; 22 Nov., 1 in 2000 + +; 29 Nov., 1 in 2000 +; 13 Jan., 1 in 2000 + + +; 21 Feb., 1 in 500 +.

Faeces: Exams. on 1 Nov., 14 Nov., 17 Nov., 23 Nov., 1 Dec., 12 Jan., 4 Feb., 21 Feb. All negative.

Rabbit VI. Wt. 2.68 kilos. Inoc. Oct. 22, 1910.

Agglutinins: 31 Oct., 1 in 2000 + + +; 9 Nov., 1 in 2000 + + +; 22 Nov., 1 in 2000 + + +; 29 Nov., 1 in 2000 + + +; 13 Jan., 1 in 2000 + + +; 21 Feb., 1 in 2000 +.

Faeces: Exams. on 1 Nov., 14 Nov., 17 Nov., 23 Nov., 1 Dec., 12 Jan., 4 Feb., 21 Feb. All negative.

Rabbit VII. Wt. 2.0 kilos. Inoc. Oct. 24, 1910.

Agglutinins: 1 Nov., 1 in 2000 + + +; 10 Nov., 1 in 2000 + + +; 22 Nov. 1 in 2000 + + +; 30 Nov., 1 in 2000 + + +.

Faeces: **Positive** on 4 Nov. 1910.

Further examinations on 14 Nov., 19 Nov., 28 Nov., 1 Dec. were all negative.

Animal died on 6 Jan. 1911 (see below).

Rabbit VIII. Wt. 2.65 kilos. Inoc. Oct. 25, 1910.

Agglutinins: 1 Nov., 1 in 2000 + + +; 10 Nov., 1 in 2000 + + +; 22 Nov., 1 in 2000 + + +; 30 Nov., 1 in 2000 + + +; 24 Jan., 1 in 500 + +, 1 in 2000 O; 23 Feb., 1 in 500 + +.

Faeces: Exams. on 4 Nov., 14 Nov., 19 Nov., 28 Nov., 1 Dec., 18 Jan., 6 Feb., 22 Feb. All negative.

Rabbit IX. Wt. 2.35 kilos. Inoc. Oct. 26, 1910.

Agglutinins: 1 Nov., 1 in 2000 + + +; 10 Nov., do.; 28 Nov., do.; 6 Dec., do.; 24 Jan., 1 in 2000 +; 23 Feb., 1 in 2000 +.

Faeces: Exams. on 4 Nov., 15 Nov., 19 Nov., 28 Nov., 5 Dec., 18 Jan., 6 Feb., 22 Feb. All negative.

Animal showed paralysis of hind legs and diarrhoea and was killed (see below).

Rabbit X. Wt. 2.85 kilos. Inoc. Oct. 27, 1910.

Agglutinins: 7 Nov., 1 in 2000 + + ; 10 Nov. 1 in 2000 + + + ; 28 Nov. 1 in 2000 + + ; 6 Dec., do. ; 24 Jan., 1 in 2000 +.

Faeces: **Positive** on 4 Nov. 1910.

Further examinations on 15 Nov., 19 Nov., 28 Nov., 5 Dec., 18 Jan., 6 Feb. were all negative.

Animal died on 13 Feb. 1911 (see below).

Rabbit XI. Wt. 1.97 kilos. Inoc. Oct. 28, 1910.

Agglutinins: 1 Nov., 1 in 2000 + + + ; 15 Nov., do. ; 28 Nov., do. ; 6 Dec., do. ; 24 Jan., 1 in 2000 + ; 23 Feb., 1 in 500 + + , 1 in 2000 O.

Faeces: Exams. on 9 Nov., 15 Nov., 23 Nov., 28 Nov., 5 Dec., 6 Feb., 22 Feb.

All negative.

Rabbit XII. Wt. 2.17 kilos. Inoc. Oct. 29, 1910.

Agglutinins: 7 Nov., 1 in 2000 + ; 15 Nov., 1 in 2000 + + + ; 28 Nov., do. ; 6 Dec., do. ; 25 Jan., do. ; 23 Feb., 1 in 2000 +.

Faeces: Exams. on 9 Nov., 15 Nov., 23 Nov., 28 Nov., 5 Dec., 13 Feb., 22 Feb.

All negative.

In two instances only, therefore, was the typhoid bacillus recovered from the faeces, at the end of eight days after inoculation in the case of Rabbit X and at the end of 11 days in the case of Rabbit VII. Rabbit VII was found dead on 6 Jan. 1911, *i.e.* about $2\frac{1}{2}$ months after inoculation. The *B. typhosus* was recovered in pure culture from the bile and gall-bladder wall but not from the heart blood or colon. Plate III shows a cross section of the gall-bladder in this case. Apart from the gall-bladder lesion and the finding of *B. typhosus* no obvious cause of death could be found. Although the heart blood gave a negative result, the possibility is not excluded that death may have been due to an autoinfection. One has also to consider the possibility of some anaphylactic disturbance as a contributory cause of death owing perhaps to the sudden liberation of typhoid antigen from the gall-bladder area. Dr Ledingham informs me that on several occasions he has isolated *B. typhosus* from the gall-bladder of guinea-pigs which had received sublethal doses of the living organism 4-5 weeks previously.

Rabbit X was found dead on 13 Feb., 1911. There were no obvious lesions at autopsy and the *B. typhosus* was not recovered. (Examination of Bile, Gall-bladder wall, Duodenum, Ileum, Colon, Spleen, Liver and Heart Blood.)

Rabbit IX was killed after showing paralysis of the hind legs and diarrhoea. *B. typhosus* was not recovered from any of the organs.

Attempts to exhibit B. typhosus in the faeces by the use of purgatives.

Owing to the persistently negative results obtained by examination of the faeces in the majority of the rabbits, it was thought that the administration of purgatives might help matters. The rabbits however proved to be extraordinarily resistant to nearly all the well-known purgatives.

The following drugs were given by the mouth, one after the other without effect: Mag. Sulph. $\frac{1}{2}$ oz., Phenolphthalein gr. XV, Pulv. Jalap. Co. $\frac{1}{2}$ oz., Paraffinum liquidum $1\frac{1}{2}$ oz., and finally Croton oil in doses of 13 minims.

Arecolin $\frac{1}{10}$ grain was given hypodermically with no better result, also $\frac{1}{100}$ grain of Eserine, the only effect of which was to produce violent spasms in the hind legs, lasting for about two hours but which led to no purgation. At last it was found that Calomel in doses of 4 grains produced slight purgation on the following day but not invariably. A dose of Calomel was therefore given to each rabbit on the day before the faeces was collected but in spite of the purgation it was impossible to recover the typhoid bacillus.

Laparotomy and Puncture of Gall-bladder.

Between Feb. 28, 1911, and Mar. 9, 1911, laparotomy was performed on the remaining seven rabbits but in four only was the gall-bladder found infected at the operation. In all four cases the bile, removed by puncture, proved sterile. One cannot conclude, however, that the animals are uninfected as it has been shown that the bile may be free from typhoid bacilli while the bladder wall may still yield a positive result on culture.

All the animals made an excellent recovery from the operation and it is proposed to reinoculate them in order to determine whether, according to Fornet's view (see Ledingham, 1910), the carrier-state is more likely to follow a second infection.

The blood of these 7 rabbits was still found to agglutinate the typhoid bacillus, four months after injection in a dilution of 1 in 500.

SUMMARY.

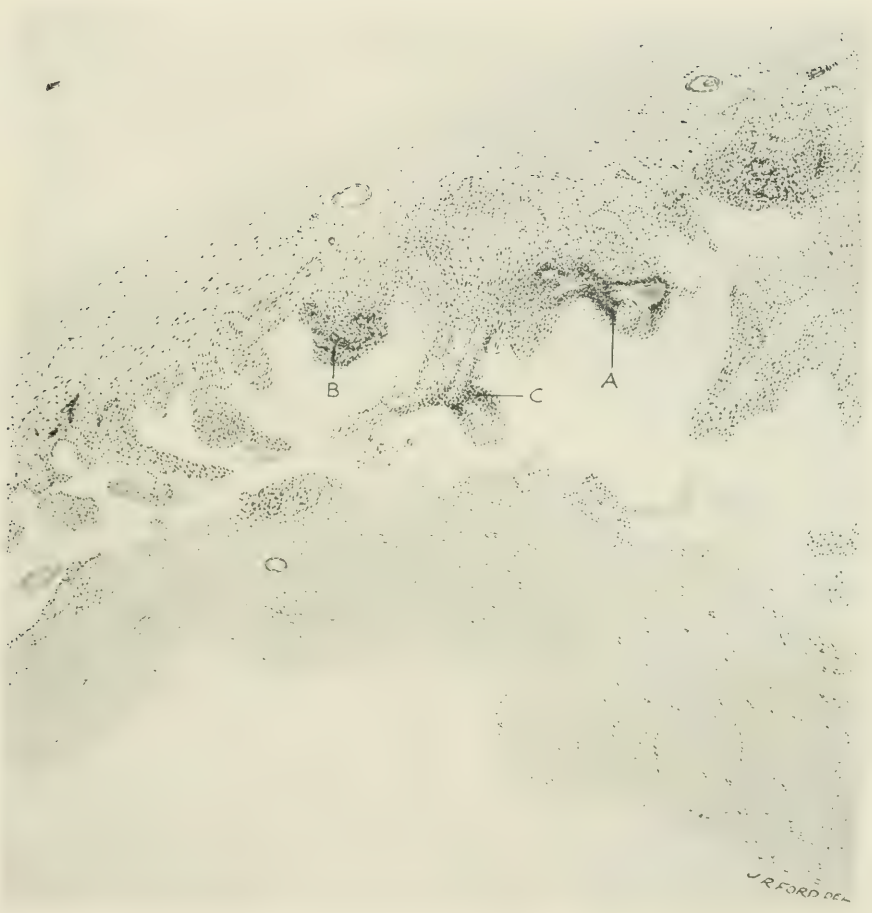
Preliminary record of attempts to produce the typhoid-carrier state in the rabbit and to obtain evidence of the presence of this condition by bacteriological examination of the faeces.

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DESCRIPTION OF PLATE III.

Section of mucosa of gall-bladder (Rabbit VII), from which *B. typhosus* was recovered at death $2\frac{1}{2}$ months after inoculation. Rugose condition well marked. Inflammatory foci with numerous chromatic debris (A, B, C) present in some of the papillae, and in one of them (A) the focus surrounds the central vessel of the papilla. Organisms were not definitely recognised in these foci by staining methods. These partially necrotic foci obviously represent the advanced stage of the embolic bacillary dépôts observed in early cases.



Illustrating the paper by Mr H. de R. Morgan.
(Description: see p. 207.)

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ON THE VARIETIES OF *BACILLUS COLI* ASSOCIATED WITH THE HOUSE-FLY (*MUSCA DOMESTICA*).

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THE importance of flies in relation to the transmission of infectious diseases has been manifested in numerous instances; the incrimination of the house-fly, however, is as yet based upon by no means irrefutable grounds. Many observations have been made which must arouse grave suspicion, but the evidence hitherto adduced is far from conclusive. That house-flies can, and do convey infective material has been demonstrated both experimentally and under natural conditions, but the full significance of the house-fly as a disease-transmitter and the circumstances under which it assumes this rôle are still vague and indefinite.

The relation, in particular, of the house-fly to bacteria, pathogenic and otherwise, is not so thoroughly understood as might be desirable. It is well known that it may harbour a considerable variety of bacteria in its alimentary canal and carry an equal variety on the surface of its body, yet it is quite uncertain what organisms, if any, are particularly associated with the fly. It is not to be doubted that the bacterial flora of the fly's intestine may change under certain circumstances; at one time a particular group of organisms may be abundant and be replaced by others at some later period. The mutual relationship of these bacteria and their attitude towards organisms introduced into the intestine, are factors which must be considered in dealing with the problem of the house-fly as a carrier of pathogenic bacteria.

Only a very limited number of bacteriological examinations of flies under natural conditions has hitherto been made, and there has been no attempt to furnish a detailed account of such bacteria as have been

found. One of the earliest investigators to consider the relation of bacteria to flies was G. Marpmann (1884), who examined 230 flies. His method was to press a drop of fluid from the proboscis and from the anus, and to examine these in stained preparations. He determined the presence of bacteria in every one of the flies, but beyond this he did not go. In 1903 W. H. Horrocks examined a number of flies in which he found lactose-fermenting bacilli which he considered to be *B. coli communis*, as they did not ferment saccharose. Graham-Smith (1909) made a series of examinations of flies for the presence of colon bacilli and classified the bacilli so found according to MacConkey's four groups. He found that out of 94 house-flies 21 % carried such bacilli, but only 4 % (out of 56) contained them in their intestine.

The present investigation, which is only preliminary in nature, follows the lines adopted by Graham-Smith, but the characters of the organisms isolated have been studied in greater detail in accordance with the methods proposed by MacConkey (1909). Many of the flies examined were obtained from houses in which epidemic diarrhoea had occurred¹, but various other sources were also drawn upon.

It has been shown very clearly by Graham-Smith (1910) that although many bacteria may adhere to the external surface of flies, they do not, in the case of non-sporing forms, survive there for more than a few hours. On the other hand, bacteria which have been ingested by the fly may remain in its intestine and be passed in its faeces for periods reckoned in days. The intestine must therefore be regarded as a more important vehicle of transit than the legs or surface of the body. In his experiments Graham-Smith found that, by feeding flies on pure cultures, *B. typhosus*, *B. enteritidis* (Gaertner) and *Vibrio cholerae* could be recovered from the intestine 6, 7, and 2 days later, respectively. Ficker (1903), Hamilton (1903) and Faichnie (1909a) have isolated typhoid bacilli from the intestine of flies caught in the vicinity of patients suffering from enteric fever. Faichnie (1909b), moreover, was able to recover typhoid bacilli from a fly which had become infected 16 days previously. These experiments and observations show that the typhoid bacillus may remain in the fly's intestine for a considerable period even under natural conditions.

Graham-Smith came to the conclusion that typhoid bacilli do not grow or multiply within the fly's intestine, and that they are more or less rapidly eliminated or destroyed. My own experiments in this

¹ I have to thank Dr G. Quin Lennane, M.O.H. Battersea, for affording me the opportunity.

respect lead to the same conclusion. That other bacteria, however, amongst which some pathogenic to man, may grow and multiply inside the fly, is not without the range of possibility. During the course of my experiments, I have found that certain varieties seem to be able to establish themselves in the fly's intestine, to the exclusion, sometimes, of other forms, and to remain there for a considerable time. Amongst these may be mentioned Morgan's *Bacillus* No. 1 and *Bacillus paratyphosus* β . The former I have met with not infrequently and a few flies have yielded almost a pure culture of this organism. *B. paratyphosus* β I have found in two flies, which were being used for experimental purposes. They formed part of a batch of flies which were fed on a sample of faeces from a typhoid carrier. These two particular flies were examined 4 and 7 days respectively after they had fed on the faeces. *B. paratyphosus* β was recovered from the external surface and intestine of the first fly and from the intestine of the second, in each case in almost pure culture. The identity of the bacillus was confirmed by Dr F. A. Bainbridge. Bacteriological examination of the faeces on which the flies had fed failed to reveal the presence of *B. paratyphosus* β . Three subsequent examinations, as well as many previous examinations of faeces from the same patient gave negative results, so the conclusion cannot be avoided that the flies had become infected previous to the experiment. The flies had been kept under sterile conditions for a week before experiment. The source of infection could not be ascertained but it is apparent that the second fly must have carried the bacillus for at least 14 days, or that it had become infected from the first fly, which had carried it for at least 11 days. The point of importance is that at the end of that period the bacilli were still present in large numbers in the intestine. This, so far as I am aware, is the only record of this bacillus occurring naturally in flies. Morgan's bacillus No. 1 was obtained from flies by Morgan and Ledingham (1909) and appears to be a not uncommon inhabitant of the fly's intestine.

The numerous observations which have already been made on the subject clearly establish the fact that the house-fly may carry an enormous number and a great variety of bacteria and may inoculate those into materials on which it feeds. They have also shown that many of the bacteria pathogenic to man and animals may be carried in this way. It remains to determine the extent to which this means of transmission may occur in nature. To this end one of the most essential points appears to be a knowledge of the natural relation of flies to bacteria. A knowledge of the bacteria naturally occurring in flies,

their relation to each other and their relation towards other, possibly pathogenic, bacteria, which may from time to time be introduced, can hardly fail to be of importance in dealing with the subject of fly-borne disease. It is evident that, before any general conclusions can be drawn from experimental work with flies in their relation to the transmission of pathogenic bacteria, or from chance observations on the natural occurrence of such bacteria in flies, the above mentioned points will require some consideration.

With regard to the natural intestinal flora, it is certain that all the bacteria which may be isolated from a fly under ordinary circumstances cannot be regarded as constituting its natural flora. Feeding as it does continuously or intermittently on contaminated material, its intestine contains a large number of bacteria derived from its food. It is more than likely that a considerable proportion of these are simply passing through and never become resident. This becomes apparent when flies are fed for a length of time on sterile food. The characteristically faecal bacteria, such as the colon bacilli, tend to disappear under such circumstances and to be replaced by other organisms, frequently non-lactose-fermenting. The common occurrence of such organisms towards the end of experiments has given rise to the suspicion that they perhaps represent to some extent the natural intestinal flora of the fly. This suspicion is strengthened by the observation of Graham-Smith (1911) that non-lactose bacteria commonly occur in recently emerged flies. Dr Ledingham has also had a similar experience in a series of experiments, the results of which are in course of publication¹. I am indebted to him for his information on this matter.

The majority of the flies examined in the present investigation were obtained from the dwelling rooms of houses in London. Several were also examined from the various outhouses in connection with the laboratory. The procedure was almost invariably the same. The flies were first well washed in sterile broth, then in 2% lysol or absolute alcohol for 10-20 minutes. They were then thoroughly washed in sterile water, dried over a flame and the whole alimentary canal was placed in broth. After incubation at 37° C. overnight the broth cultures were plated on MacConkey's bile salt medium, and from each plate about a dozen colonies were picked off and their characters determined. Altogether 145 specimens of *Musca domestica* were examined. 25 of these were examined individually, the rest in 23 lots of 5 to 7 each. Of the 96 plates which were made from these, 55 showed

¹ *Vide Journ. of Hyg.* xi. No. 3, 1911.

the presence of lactose-fermenting bacilli. 21 external cultures and 20 internal were negative. In 12 cases lactose-fermenting bacilli were found neither inside nor outside. Less than 75 % of the flies may therefore be regarded as carrying colon bacilli either in their intestine or on the surface of their body. In 3 cases no growth of any kind was obtained from the surface or the intestine. In 9 cases there was a growth from the surface but not from the intestine, and in 5 cases a growth from the intestine but not from the surface. At least 5 % therefore of the flies carried no aerobic bacteria capable of growing on MacConkey's medium.

Excluding the numerous cases in which two or more colonies of the same organism were picked off the same plate, colon bacilli were met with 77 times. These represented 27 different varieties and most of them possessed characters corresponding with those of some organism in MacConkey's list. Seven varieties not agreeing with any in that list were met with, but without exception only in isolated cases.

The chief difficulty encountered in classifying these organisms was with regard to the liquefaction of gelatine. The great majority have shown no trace of liquefaction at the end of a year. Some, however, have shown the first signs after six months. Only a few produced marked liquefaction within four months. The latter proved to be *Bacillus cloacae*, or Nos. 36 or 102 in MacConkey's list.

The indole test was performed twice in every case and found to be constant. The Voges and Proskauer's test was repeated in several doubtful cases. The motility was always investigated in a six hours' broth culture. The sugar fermentation reactions were found to be remarkably uniform. With only one exception, acidity and gas production were apparent within 48 hours in the case of glucose, mannite, saccharose and dulcitol, and in the negative cases, no further change was noticed at the end of six days. The exception was a strain of *B. vesiculosus* which fermented dulcitol after four days. With regard to lactose, fermentation was almost invariably slower, but only in two cases was it delayed beyond the fourth day. The same applies to adonite. With regard to inulin the result was negative even at the end of ten days except in one case, in which fermentation occurred within four days.

The following table shows the characters and numbers of the organisms investigated. They were all Gram-negative, gave acid and gas in lactose, glucose and mannite and produced acid and clot in litmus milk. They are numbered and named according to MacConkey's list.

TABLE.

TABLE.											Numbers obtained				
				Gelatine	Motility	Indole	Saccharose	Dulcite	Adonite	Inulin	Voges and Proskauer	External	Internal	Total	
GROUP I.															
1.	...	...	...	...	-	+	+	-	-	+	-	-	1	1	2
2.	<i>B. acidi lactici</i>	...	...	...	-	-	+	-	-	+	-	-	0	1	1
4.	<i>B. Grünthal</i>	...	...	...	-	+	+	-	-	-	-	-	3	2	5
5.	<i>B. vesiculosus</i>	...	...	...	-	-	+	-	-	-	-	-	2	0	2
7.	...	...	...	...	-	+	-	-	-	-	-	-	2	2	4
8.	<i>B. coli mutabilis</i>	...	...	...	-	-	-	-	-	-	-	-	2	2	3
				Total								10	8	17	
				Number of varieties								5	5	6	
GROUP II.															
34.	<i>B. coli communis</i>	...	...	...	-	+	+	-	+	-	-	-	5	10	14
35.	<i>B. Schaffer</i>	...	...	...	-	-	+	-	+	-	-	-	1	1	2
36.	...	...	...	...	+	+	-	-	+	-	-	-	1	0	1
33a.	...	...	...	...	+	+	+	-	+	+	-	-	0	1	1
36a.	...	...	...	...	-	+	-	-	+	-	-	-	1	1	1
				Total								8	13	19	
				Number of varieties								4	4	5	
GROUP III.															
65.	<i>B. oxytocus pernicius</i>	...	...	...	+	-	+	+	+	+	+	+	0	1	1
66.	...	...	...	...	-	-	+	+	+	+	-	-	0	1	1
67.	...	...	...	...	-	-	-	+	+	+	-	+	1	2	2
71.	...	...	...	...	-	+	+	+	+	-	-	-	6	7	11
72.	<i>B. Neapolitanus</i>	...	...	...	-	-	+	+	+	-	-	-	2	0	2
74.	...	...	...	...	-	+	-	+	+	-	-	-	0	1	1
75.	...	...	...	...	-	-	-	+	+	-	-	+	1	1	2
66a.	...	...	...	...	-	+	+	+	+	+	-	-	0	1	1
				Total								10	14	21	
				Number of varieties								4	7	8	
GROUP IV.															
101.	...	...	...	...	-	-	+	+	-	+	-	-	0	1	1
102.	...	...	...	...	+	+	-	+	-	+	-	+	2	4	6
104.	<i>B. gasiformans non-liquefaciens</i>	...	...	...	-	-	-	+	-	+	-	-	2	2	3
106.	...	...	...	...	-	+	+	+	-	-	-	-	1	2	3
108.	<i>B. cloacae</i>	...	...	...	+	+	-	+	-	-	-	+	0	3	3
109.	...	...	...	...	-	+	-	+	-	-	-	-	1	1	2
106a.	...	...	...	...	+	+	+	+	-	-	-	-	0	1	1
108a.	...	...	...	...	+	+	+	+	-	-	-	+	1	0	1
				Total								7	14	20	
				Number of varieties								5	7	8	
				Total in all the groups								35	49	77	
				Total number of varieties								18	23	27	

Comparing the above table with that given by MacConkey (1909) it is apparent that a considerable similarity in respect of the colon bacilli exists between the bacterial flora of flies, and the bacteria met with in the faeces of man and other animals. The most striking feature is the marked preponderance of the characteristic faecal organisms *B. coli communis* and MacConkey's bacillus No. 71.

From the varied sources from which his organisms were derived, MacConkey found that those belonging to group III were greatly in excess of those belonging to the other groups and constituted 45% of the whole. Those of group IV were only about a third as frequent. From purely faecal sources the disproportion between these two groups is still more marked. In a general way, therefore, it may be considered that the bacilli of group III are more characteristically faecal organisms than those of group IV. The latter were obtained for the greater part from non-faecal sources. The varieties in groups I and II were more frequent in faeces than in other materials. Again in human faeces groups I, II and III were all about equally common, while group IV was comparatively rare. An examination of my table will show that each of the groups is about equally represented. There is not the comparative rarity of group IV characteristic of excremental material nor the rarity of groups I and II characteristic of other sources. This would indicate that the colon bacilli found in flies comprise a mixture derived both from faecal and other sources. Such a conclusion is in accord with preconceived notions.

With regard to the individual varieties, those most frequently met with by MacConkey were No. 71, No. 34 (*B. coli communis*) and No. 5 (*B. vesiculosus*), and these were the three organisms which occurred most frequently in excremental matter. They were also most frequent in human excrement. From other sources, however, the most common varieties were Nos. 71, 73, and 108 (*B. cloacae*). *B. coli communis* and *B. vesiculosus* were much rarer. Now in *Musca domestica* the most common varieties were 34 and 71 followed by 102, 4 and 7. The great frequency of *B. coli communis* can hardly be regarded as other than direct evidence of faecal contamination. The same may be said of No. 4, which was never found by MacConkey apart from faeces.

It is impossible to tell to what extent the flies in the present investigation have acquired contamination from human excrement. The number of bacterial organisms which have from time to time been isolated from human faeces and which appear capable of living for a longer or shorter period in the human alimentary canal is very great.

The same holds good in the case of several animals, and from the very incomplete evidence at present in existence, the intestinal flora of various mammals have very much in common. Differences and distinctive features have however not passed unnoticed, and with fuller knowledge the presence of a certain few bacteria might be sufficient to warrant the diagnosis of excremental matter from a particular animal or class of animals. This would demand more intimate acquaintance with the bionomics of bacteria, and in particular of those which are parasitic in man and animals. Modern methods have shown that bacteria under artificial conditions have very varied physiological requirements, and there is no reason to suppose that their requirements under natural conditions are any less varied. If such is the case, it follows that for each class or group of bacteria and for individual species or varieties of that group, there will be some environment which is an optimum and in which that particular species thrives and develops to the fullest extent. There can be no question that a great variety of physiological environment does exist in the alimentary canal of animals. Some organisms are known to require an extremely specialised environment, while others can accommodate themselves to a wide variety. For this reason the distribution of some organisms is extremely wide, while that of others is restricted. From biological considerations it may be assumed that each variety of environment will call forth the existence of a corresponding variety of organism and that the latter is conditioned by the former. There should therefore be just as many species or varieties of organisms as there are variations in environment. The different environments obtaining in the intestine of man and other animals should be sufficient to differentiate distinctive groups of bacteria and these groups would probably be readily isolated in the faeces were it not for the fact that a large number of bacteria probably simply pass through the intestine with the food, not actually growing and multiplying, but at the same time not being killed off in the process. It ought however to be possible by suitable experiment to elucidate this matter and I hope to be able to do so to some extent in the case of flies.

SUMMARY.

1. A study of the natural bacterial flora of the house-fly appears to be essential in forming a correct estimate of the part played by flies in transmitting pathogenic bacteria.
2. The house-fly may carry at least 27 varieties of *Bacillus coli*, by

far the most frequent of which are *B. coli communis* and MacConkey's bacillus No. 71.

3. As far as can be judged from the character of these colon bacilli the house-fly derives its bacterial flora equally from excremental matter and from other sources.

4. Certain non-lactose fermenting bacilli appear to be capable of multiplying in the intestine of the house-fly. Of these Morgan's bacillus No. 1 is a not infrequent inhabitant of the fly's intestine and *B. paratyphosus* β has been found on two occasions.

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FURTHER EXPERIMENTS ON VARIABILITY IN THE GAS-FORMING POWER OF INTESTINAL BACTERIA.

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(With 5 Diagrams and 2 Charts.)

IN a recent communication (Penfold 1911) I described experiments with three organisms, *B. coli* Escherich, *B. enteritidis* Gaertner and *B. Grünthal*, showing that limitation or complete abolition of certain gas-forming powers normally possessed by these organisms, could be brought about by their growth on chloracetic acid-agar, and further, that in the case of the latter organism, resistance to this medium was associated with such limitation or abolition.

In this paper I desire to give an account of further experiments dealing with the same subject. The same special medium and technique were employed as detailed in the paper cited above. Further experiment had shown that *B. lactis aerogenes* when grown for seven successive generations on chloracetic acid-agar, still gave at the end of the period a good yield of gas on glucose, although it was highly resistant to the chloracetic acid medium; similarly a strain of *B. acidi lactici* gave negative results in spite of long training.

In view of the anomalous behaviour of these organisms it appeared desirable to examine more fully the nature of the selective process in those cases in which non-gas-producing variants had been so easily obtained.

Further experiments with B. coli Escherich.

Chloracetic acid-agar plates of this organism show, at suitable concentrations of the salt, mixtures of big and little colonies, that is, the growth of all the organisms is not inhibited equally; at higher concentrations only the big colonies grow. In the successful experiments described in my first paper, the non-gas-producing strains were

obtained by selecting big colonies in each case. Further work has shown me that the *association of non-gas-production and resistance to chloracetic acid* is in the case of *B. coli* Esch. a very variable one. Some essential connection exists between the two properties, but this connection could be disclosed only by a prolonged statistical enquiry. The following experiment illustrates this point:

A pure culture of normal *B. coli* Esch. was plated out on 12 plates of chloracetic acid-agar. These plates were divided into four groups containing different concentrations of sodium chloracetate, each member of a group of three containing the same concentration of this salt.

The respective concentrations were 0.01 %, 0.05 %, 0.1 % and 0.2 % expressed in terms of the amount of acid added. The three plates of the latter concentration showed only large colonies. Five large colonies from each of the 0.2 % plates were inoculated into glucose-peptone-water in Durham tubes; these tubes were observed daily for five days and the maximum amounts of gas found were as indicated in the following table:

TABLE I.

Colonies from plate D	1/3	1/3	1/3	1/2	5/12
" " H	1/4	nil	1/2	1/2	1/2
" " L	7/12	7/12	5/12	1/2	1/2

These fractions indicate the length of gas tube displaced by gas.

Control tubes of glucose-peptone-water, inoculated from the same original strain of *B. coli* grown on MacConkey plates, gave in the case of twenty different colonies the following volumes of gas:

4 tubes yielded gas equal to 1/2 of the gas tube.									
12	"	"	"	"	"	7/12	"	"	"
4	"	"	"	"	"	2/3	"	"	"

The colonies from the chloracetic agar plates, therefore, show one giving no gas, six others below the lowest of the controls, and eight similar to the controls.

The strain from plate H, which gave no gas on glucose-peptone-water, yielded, when tested on lactose-peptone-water, 1/12th of a tubeful of gas. It was therefore plated out on a lactose plate and eight of its colonies were tested again on lactose-peptone-water, when seven of these were found to produce no gas while the remaining one showed half-a-tubeful. The large colonies, apparently, do not appear to be homogeneous in their composition.

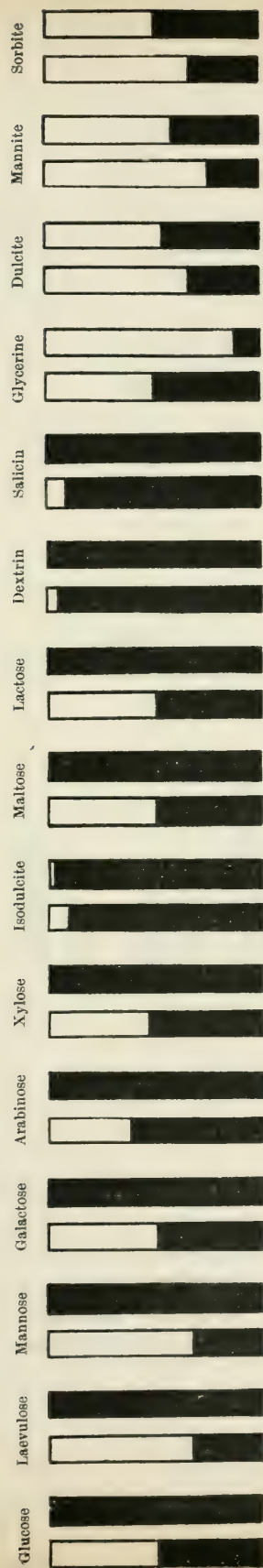


Diagram I. *Bacillus coli communis* Escherich, strain H. Left-hand member of couple = normal strain; right-hand member of couple = variant strain. In this diagram the black columns represent gas tubes containing no gas, while in the other columns the white spaces represent the quantities of gas formed. All the tubes were incubated for five days at 37° C., and the amount of gas indicated in the diagram is the *greatest* amount found in the individual tubes during the whole period. The tubes are arranged in couples, the left-hand member representing the normal *Bacillus coli*.

One of the seven lactose broths which gave no gas (denoted by *H*) was subcultured on agar and tested on the usual peptone-waters containing different carbohydrates. Diagram I represents its behaviour in respect of gas-formation on the carbohydrates which it fermented.

This strain "*H*" is a more striking example of the loss of the power to form gas from sugars, than the strain which the writer described in the formerly published paper dealing with gas variants. In the former case the organism still produced traces of gas from galactose, xylose, arabinose, isodulcitol, maltose and salicin, whereas this new strain produced only a slight trace of gas in isodulcitol, out of nine sugars tested. The essential difference between the selective processes in the two cases was, that in the former strain a large colony was taken off the first chloracetic agar plate haphazard, and replated on the same medium, from which again a single colony was picked off and replated, the process being repeated four times, while in the case of the latter strain, a large number of colonies were tested from the first chloracetic agar plates, with the result that one was found which from the start failed to give any gas on

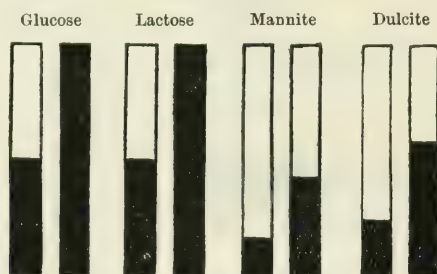


Diagram II. *B. coli* Escherich. Left-hand member of couple = normal strain; right-hand member of couple = variant strain.

glucose. In order to make certain that this was not a matter of chance, the same method was repeated again with the same organism. In this case 17 colonies were inoculated into glucose broth, from chloracetic agar plates which had been incubated five days at 37° C. All the broths showed a full acid reaction after 24 hours, but after 48 hours two of the tubes showed no gas. On this date one of the two tubes got broken but the other showed no gas throughout an observation period of six days. This new strain gave the reactions shown on Diagram II. It would appear, therefore, that the easiest and best way to get these variants is by testing a large number of colonies from the first chloracetic agar plates.

The examination of the new variety in Theobald Smith's tubes.

The above-mentioned strain "H" was compared with the normal strain from which it was derived, in Smith's tubes, and the results obtained are recorded in Table II. The new strain, it will be seen, gave no gas in glucose but its yield in mannite broth was only 50% of the normal, a diminution distinctly greater than was found with the Durham tube (see Diagram I).

TABLE II.

*Theobald Smith's tubes.*Strain "H" versus Normal *B. coli* Esch.

Normal <i>B. coli</i> .				Varied <i>B. coli</i> .			
Glucose				Glucose			
1st tube with 20 divisions		2nd tube with 10 divisions		Tube with 20 divisions		Tube with 10 divisions	
Vols. of gas 1st day 10 div.		4·8 div.		nil		nil	
,, 2nd ,, 11 ,,		4·8 ,,		nil		nil	
,, 3rd ,, 11 ,,		4·8 ,,		nil		nil	
,, 5th ,, 10 ,,		4·2 ,,		nil		nil	
Mannite				Mannite			
Tube with 20 divisions		Tube with 5 divisions		Tube with 20 divisions		Tube with 5 divisions	
Vols. of gas 1st day 8·5 div.		2·2 div.		4·5 div.		1·4 div.	
,, 2nd ,, 13·5 ,,		3·4 ,,		7·0 ,,		2·1 ,,	
,, 3rd ,, 14·5 ,,		4·0 ,,		7·2 ,,		2·1 ,,	

Further experiments with B. enteritidis Gaertner.

The variant strain of *B. enteritidis* Gaertner described by the author in a former paper, while unable to produce gas from certain pentoses, was still able to produce a little gas from the hexoses. In order, therefore, to obtain strains unable to produce gas from glucose, it was deemed advisable to test a large number of colonies from the first chloracetic agar plates in respect of their action on glucose broth.

A typical strain of *B. enteritidis* was plated on the special medium on a series of plates: the strength of chloracetic acid in these plates varied from 0·1% to 0·9%. These plates were grown for three days at 37° C. when colonies of large size were picked off and inoculated into glucose broth in order to test their gas-forming power. The results are given in Table III.

TABLE III.

Strength of chloracetic acid in the plates	Number of large colonies tested	Extreme deviation of gas yield, in fractions of gas tube
0.1 %	4	1/3 to 5/12
0.3 %	4	1/4 to 5/12
0.4 %	4	1/3 to 5/12
0.5 %	4	1/12 to 1/3
0.7 %	4	1/6 to 5/12
0.9 %	4	1/4 to 5/12

Here we have 24 colonies none of which give less than one-twelfth of a tubeful of gas. The same plates were, therefore, further incubated until the tenth day, when the large colonies were again inoculated into glucose-peptone-water and gave the gas yields seen in Table IV.

TABLE IV.

Strength of chloracetic acid in the plates	Number of large colonies tested	Extreme deviation of gas yield, in fractions of gas tube
0.1 %	8	1/12 to 5/12
0.3 %	8	Pin point bubble to 1/12
0.5 %	8	" " " 5/12
0.7 %	8	Nil to 5/12
0.9 %	8	Nil to 1/12

It appeared, therefore, that between the third and the tenth day the character of these colonies had altered, the organisms of which they were composed having become distinctly poorer gas producers. The gas-producing elements in the colonies had probably been killed off or outgrown by the non-gas-producing ones. The fact remains that no less than three of the plates showed big colonies able to produce amounts of gas nearly equal to those produced by the normal strains. Out of the total of 40 colonies tested, only three showed themselves unable to produce gas in glucose broth, while no less than six gave an amount of gas just equal to a pinhead bubble. Three tubes out of the 40 showed 5/12ths of a tubeful of gas, *i.e.*, an amount but little different from the normal. It is important to notice, that in spite of these very different gas yields, all the colonies agreed in having grown to a large size on the special medium. Two of the three glucose tubes which gave no gas had subcultures taken from them on to agar slopes. From the latter they were put through the different carbohydrate broths with results shown in Diagram III.

Both these new strains produced no gas on either glucose or maltose but still gave very fair yields from the alcohols; the retention of the slight gas-producing power of xylose is rather curious, and in this respect they differ from the strain of which an account was published in my previous communication.

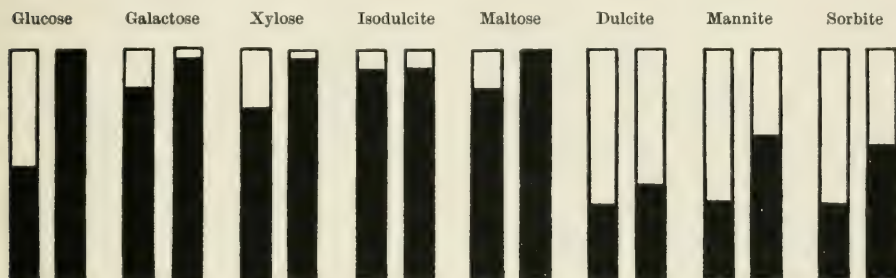


Diagram III. *Bacillus enteritidis* Gaertner. Left-hand member of couple = normal strain; right-hand member of couple = variant strain.

Further experiments with B. paratyphosus B.

A standard laboratory strain of this organism was plated on chloroacetic acid-agar plates, in which the strength of chloroacetic acid varied from 0.1% to 0.9%. Large and small colonies developed. After three days' growth 14 large colonies from the plates were tested on glucose-peptone-water. All showed some gas production. After seven days' further incubation 20 large colonies were tested on glucose-peptone-water, when no less than 14 of them were found to produce no gas. Five of these were put through various carbohydrates, but none of



Diagram IV. *B. paratyphosus* B. Left-hand member of couple = normal strain; right-hand member of couple = variant strain.

them was able to produce gas from glucose or maltose. One of the strains tested on galactose produced no gas while they all produced gas from the alcohols they fermented. An idea of the amount of gas production on alcohols is given in Diagram IV in the case of one of the strains. The others were very similar.

The occurrence of natural variants of the Gaertner group in respect of gas formation.

I am indebted to Dr Bainbridge for numerous strains belonging to this group, two of which deserve mention in this paper.

1st. A non-gas-producing *B. Aertryck*. This gave all the cultural and serological reactions proper to this variety, except that it failed to produce gas from any sugar.

2nd. A strain of *B. paratyphosus* B. (Murray) which produced very little gas on any medium. This organism was irregular in its action on dulcete broth but apart from this, and its poor gas-producing power, it was a definite strain of *B. paratyphosus* B. Moreover it had been isolated from a definite case of paratyphoid fever. This strain was grown on chloracetic acid-agar with a view to removing its gas-forming power, and the normal and selected strains are compared in this respect in Diagram V.

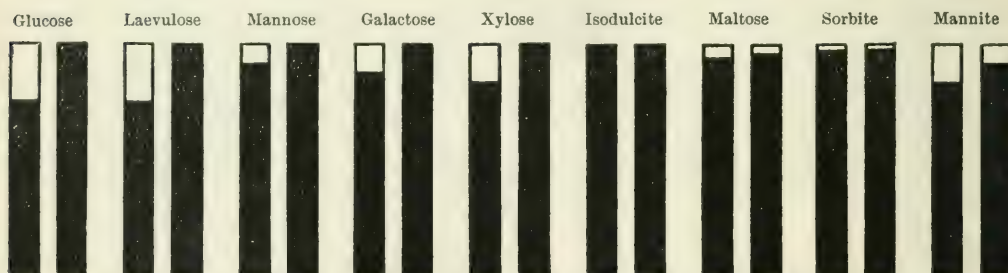


Diagram V. Strain "Murray," *B. paratyphosus* B. Left-hand member of couple = normal strain; right-hand member of couple = variant strain.

Here again the power to form gas from sugars disappeared except in the case of maltose; the power to produce gas from mannite and sorbite is retained to a slight extent, but for which fact it would have approached very closely to the naturally occurring non-gas-producing *B. Aertryck* above described. The agglutination characters of this organism were not altered in the process of selection.

Morgan's bacillus No. 1. This organism by growth on chloracetic acid-agar lost the power to produce gas from glucose and laevulose, though it produced a good acid reaction in the medium overnight. On galactose no acid reaction was produced by the new strain until the sixth day, and then, curiously enough, a tenth of a tubeful of gas appeared. This was repeated and both these effects were obtained.

It seems therefore highly probable that this selective process for obtaining gas variants will be fairly generally successful if thoroughly applied. To get successful results, it is advisable to test a large number of colonies in the first instance and to allow the plates to incubate for 7-10 days before testing the colonies.

A comparison of the gas-forming power of the big and little colonies taken from the same chloracetic-agar plates.

B. enteritidis Gaertner was plated out on a series of plates containing ascending concentrations of chloracetic acid. After three days' incubation four big and four little colonies from each plate were subcultured into glucose broth when the average yield of gas was found to be as follows. (The results are expressed as tube divisions filled with gas.)

	Big colonies	Little colonies	Ratio
0.1 % plate	4.8	6.5	0.73
0.3 "	4.25	5.6	0.75
0.4 "	4.75	5.5	0.86
0.5 "	2.4	4.1	0.58
0.7 "	3.8	6.0	0.63
0.9 "	3.0	7.5	0.4

B. paratyphosus B. treated similarly gave the following figures:

	Big colonies	Little colonies	Ratio
0.1 % plate	2.6	2.5	1.0
0.2 "	1.3	2.8	0.46
0.3 "	1.0	3.1	0.32
0.7 "	0.5	2.6	0.19
0.8 "	1.0	3.2	0.3
0.9 "	0.5	2.0	0.25

These ratios are plotted in the accompanying Charts I and II.

The figures show that even after three days' growth on the special medium the big colonies are poorer gas producers than the small ones, the latter being in point of fact normal. Twenty-four small colonies of *B. enteritidis* Gaertner gave an average of 5.8 divisions of gas against

five normal controls which average five divisions. The disparity between big and little colonies in respect of gas formation, holds much more strongly when the plates have been incubated for a longer period. The gas yield of the big colonies in the case of *B. enteritidis* Gaertner and *B. paratyphosus* B., after the plates have been incubated ten days, is roughly indicated in an earlier portion of this paper (see pp. 491-3).

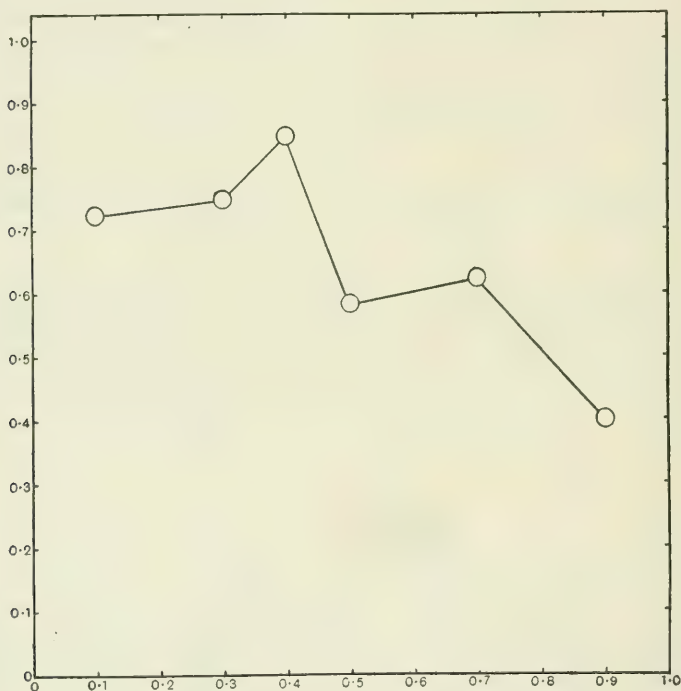


Chart I. *B. enteritidis* Gaertner. Ordinates represent ratios of average volumes of gas developed on glucose by big and little colonies respectively. Abscissae represent strengths of chloracetic acid in the plates.

Increased resistance to the chloracetic-acid medium is indicated by the increased size of some colonies, but in addition, these large colonies, in the later stages of their growth, show secondary colonies composed of organisms which appear to be more resistant to the medium than the average of the colony. In the case of *B. Grünthal*, an examination of some of these showed them to be poorer gas producers than the rest of the colony, and this agreed with the general principle that chloracetic acid resistance and poor gas-producing power are correlated. In the

case of *B. enteritidis* Gaertner, however, the papillae appear to be composed of organisms of similar gas-producing power to those found in the rest of the colony. This statement is based on the examination of about 40 tubes of glucose-peptone-water which had been inoculated, in equal proportions, from papillae and smooth interpapillary areas of colonies respectively. *B. paratyphosus* B. is similar to *B. enteritidis* Gaertner in this respect.

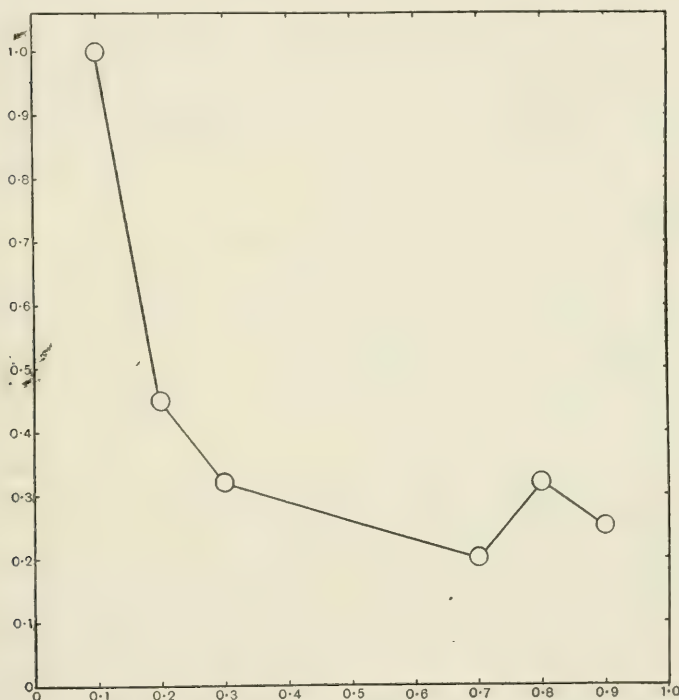


Chart II. *B. paratyphosus* B. Ordinates represent ratios of average volumes of gas developed on glucose by big and little colonies respectively. Abscissae represent strengths of chloracetic acid in the plates.

Summary of foregoing experimental results.

(1) By the method of selection indicated in the paper it has been possible to obtain a variety of *B. coli* Escherich unable to produce gas from any sugar excepting isodulcite. The new strain so obtained was still able to produce gas from all the alcohols it fermented though in diminished amount.

(2) The power of *B. enteritidis* Gaertner to form gas from glucose and maltose has been removed in the same way. *B. paratyphosus* B. and *Morgan's bacillus* No. 1 have similarly given rise to variants not producing gas from the usual sugars.

(3) The easiest method to obtain non-gas-producing variants is by incubating plates of the respective organisms on the special medium for 7-10 days at 37° C. and then testing a fairly large number of large colonies on glucose-peptone-water.

(4) The relationship of chloracetic-acid resistance to non-gas-producing power is undoubted as between the big and little colonies of various strains, but it is of a complex character. The papillae in the case of *B. enteritidis* Gaertner and *B. paratyphosus* B. do not seem to differ materially from the rest of the colony bearing them, in gas-forming power.

Durability of the new character.

In my previous communication dealing with variation of the gas-producing power of these organisms, I showed that an artificially selected strain of *B. coli* Escherich, producing no gas from glucose, evinced no tendency to reversion though subcultured frequently on glucose-peptone-water.

The period was too short, however, to furnish much information in respect of permanency. I have now observed some of the new strains for a considerable period with the following results:

1st Experiment. *B. coli* Esch. In December 1910 a new strain of *B. coli* Esch. was selected, which produced no gas from glucose but 7/12ths of a tubeful when grown in mannite-peptone-water. From this period until May 1911 the strain was grown on ordinary agar, being subcultured on it about every fortnight. On May 19th, 1911, *i.e.*, over five months after its selection, it was retested in respect of gas-producing power in glucose- and mannite-peptone-waters with the following result:

Date of observation	Glucose		Mannite	
	Reaction	Gas	Reaction	Gas
20th May	Acid	Nil	Acid	1/8
21st "	"	"	"	7/12
22nd "	"	"	"	7/12
24th "	"	"	"	7/12

2nd Experiment. Another strain of *B. coli* Esch. selected out shortly after the above, gave in May a bubble of gas the size of a pin's head when grown on glucose-peptone-water in a Durham tube.

3rd Experiment. An artificial strain of *B. coli* Esch. unable to produce gas from glucose in the Durham tube was grown anaerobically at 37° C. in glucose-peptone-water to which chalk had been added. Under anaerobic conditions it produced a small yield of gas and it was, therefore, thought to be reverting to its natural state. This anaerobic growth was continued for over one month. It was then plated out on lactose agar and three separate colonies were tested on glucose- and mannite-peptone-waters with the following result:

Glucose.

Date of inoculation, March 28.			Colony... 1		2		3	
Observed	„	29	A.f.	G.n.	A.f.	G.n.	A.f.	G. pinhead bubble
	„	31	„	„	„	„	„	„
	April	1	„	„	„	„	„	„
	„	3	„	„	„	„	„	G. bubble slightly larger

Mannite.

Date of inoculation, March 28.			Colony... 4		5		6	
Observed	„	29	A.f.	G. 1/4	A.f.	G. 1/4	A.f.	G. 1/4
	„	31	„	G. 1/2	„	G. 1/2	„	G. 1/2
	April	1	„	G. 7/12	„	G. 7/12	„	G. 7/12
	„	3	„	G. 1/2	„	G. 1/2	„	G. 1/2

A.f. = Full acid reaction.

G.n. = Gas nil.

The gas appearing in the anaerobic culture was owing therefore only in the slightest degree to reversion; it was probably largely accounted for by the anaerobic growth and the presence of the chalk.

4th Experiment. *B. Grünthal*, by growth on the special medium and selection from it, had its gas-producing power in respect of lactose reduced from half a tubeful to a pinhead bubble. The strain was grown three months on agar and then plated. Twelve colonies were tested in lactose broth, when all were found to produce the same amount of gas as the strain did when first selected out.

5th Experiment. Strains of *B. paratyphosus* B. occasionally show a tendency to revert, in respect of gas-forming power on glucose, soon after being first selected out, but this tendency does not affect all the new strains. These new varieties have not been long under observation.

Identity.

The demonstration of the identity of the new strains has not been so strictly attended to in this series as in those described in my first communication on this subject where the evidence given was very complete. Since all the new varieties described in this paper show similar qualitative changes to those described in my previous communication it seemed unnecessary to devote so much effort in this direction.

Testing of other chemical substances related to monochloracetic acid in respect of their action on the growth of intestinal bacteria.

Marked variability in the size of colonies on plates, and the development of secondary colonies (*i.e.*, papillae formation), were especially looked for.

The organic acids used were all first neutralised in aqueous solution with saturated sodium carbonate solution, then filtered through a Berkefeld and added to agar plates in the usual way. Many of these bodies are unstable so that their temperature was kept as low as possible throughout the whole work. The chemicals used were all got from Kahlbaum except the potassium chlormalonate for which I was indebted to Miss Smedley of the Lister Institute.

Monobromacetic acid. This preparation inhibited the growth of *B. coli* Esch. and *B. enteritidis* Gaertner in weaker concentrations than monochloracetic acid. In the case of both organisms distinct variability in the size of the colonies was observed in suitable concentrations, but this was not so marked as with monochloracetic acid. Papillae appeared on the colonies, well developed in the case of *B. coli* Esch., but not so well developed in the case of *B. enteritidis* Gaertner. Fifteen large colonies from *B. enteritidis* and *B. coli* plates when tested on glucose showed no loss of gas-forming power. It was not found so easy to produce races highly resistant to this agent as had been the case with monochloracetic acid.

α -Brompropionic acid. In plates containing this agent *B. coli* Esch. produced small papillae on plates having a strength of 0.05% and 0.1%. Growth was not obtained on plates having a higher concentration than 0.3%. No marked variability in the size of the colonies was noted.

B. enteritidis Gaertner and *B. paratyphosus* A. showed, on agar containing this agent, no papillae formation nor marked variability in the size of the colonies.

Colonies from brompropionic acid plates of the above three organisms showed no variations from the normal strains in amount of gas produced from glucose.

Dibromsuccinic acid and the potassium salt of chlormalonic acid failed to select out variants of *B. coli* Esch. or *B. enteritidis* Gaertner. Sodium hippurate and benzoate were tried because of Herter's statement (1909) that they caused *B. coli* to produce less gas. Six concentrations of each salt, in agar plates, were used, from 0.01 % to 2 % in the case of the sodium benzoate, and from 0.02 % to 4 % in the case of the hippurate. In the case of *B. coli* Esch. and *B. Grünthal* no marked variability in the size of the colonies or naked-eye papillae formation was noted. Colonies from the plates of highest concentrations which showed growth were inoculated on the 11th day into glucose-peptone-water and produced normal amounts of gas.

Since the publication of my first communication on this subject Revis (1911) has described the production of an artificial variety from a typical *B. coli*. This variety differed from the normal by being unable to produce gas from any carbohydrate on which it was tested; it was, moreover, unable to clot milk. It was produced by the growth of the normal strain on malachite green media. Revis does not exclude the possibility of contamination arising during the course of the 15 subcultures necessary to produce the new variety. It is precisely because of this difficulty that all points of resemblance between the original and the new strains should be collected, to help us to determine whether the new strain was actually derived from the old strain or not. I made no proviso, as Revis alleges, that before and after variation the agglutination character should remain the same. I believe however that if it does, it helps to establish the origin of the new variety. Of course variations of the agglutination property of the various organisms have been proved to occur, and the proof in this case has been largely furnished by the cultural and other characters, which have meanwhile remained constant. Had Revis proved the identity of the agglutination characters of the old and new organisms, it would have made the case more convincing. When many characters of an organism vary at once it is difficult to convince oneself that a foreign organism did not drop into the test tube during some portion of a long subculturing process and outgrow the strain from which one started. Since there are not

many remaining characters left for identification purposes, one can only produce conviction by repeating the process a number of times with the same result. I feel very little doubt about the variation process described by Revis, as it bears a strong resemblance to the one described in the previous communication I made on this subject, and in that case the identity was determined by serum tests as well as by the standard cultural tests. Moreover other organisms varied similarly in the same environment.

GENERAL CONCLUSIONS.

1. The results of this research show that organisms of the coli-group can have various gas-forming powers removed by a process of artificial selection on a specially prepared medium. The biochemical properties of the variant strains approach closely those of the naturally occurring anaerogenes-coli class.

2. The power of gas-formation from sugars (always excepting isodulcite) may be lost when gas-formation from alcohols is retained. It is probable, therefore, that two different ferments are engaged in the respective processes.

3. The research raises the question as to the weight to be attached to the power of fermenting glucose and lactose with gas-formation, in recognising *B. coli* in routine examinations of pathological material, water, foods, etc. Hitherto, in all authoritative catalogues of the necessary properties of this organism, this has been included but it probably ought to be regarded as not absolutely essential.

In conclusion I desire to express my indebtedness to Dr Ledingham for valuable help in the course of this research.

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ON THE SPECIFICITY OF BACTERIAL MUTATION, WITH A RESUMÉ OF THE RESULTS OF AN EXAMINATION OF BACTERIA FOUND IN FAECES AND URINE, WHICH UNDERGO MUTATION WHEN GROWN ON LACTOSE MEDIA.

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(Plates I to IV.)

NEISSER in 1906 claimed to have discovered an organism which, when grown on lactose-agar, threw off suddenly a new strain capable of fermenting lactose. The identity of the new strain was established by Massini (1907) by many cultural and serological tests, and from that time onwards it has been generally accepted that new bacterial strains of great permanence may arise so suddenly as to justify this type of variation being named "Mutation."

The new strain arose when the original strain was grown on lactose-agar plates. The colonies on these plates showed papillary projections, and if litmus was present the papillae showed themselves to be strongly acid in reaction while the rest of the colony showed no sign of acidity. Replating of the papillae showed them to contain a new lactose-fermenting strain. This acquired power of fermenting lactose was held with great tenacity. The new strain was unable any longer to produce papillae on its colonies when grown on lactose-agar.

Arnold Burk (1908) described a similar organism but the evidence of identification in this case was almost entirely cultural, as the strains did not produce good agglutinating sera.

Reiner Müller (1908 and 1909) showed that various other organisms produced papillary projections on their colonies on agar plates, provided that certain definite carbohydrates were added to the agar. The bacilli contained in these papillae had, in a proportion of the strains examined

acquired the power to ferment the carbohydrate. The new strains had, however, in all cases lost the power to produce papillae on the same medium, and this fact constituted the essence of the mutation processes he described.

Various interpretations have been put forward accounting for these mutation processes. The first one suggested was that of contamination. The fermenting papillae on the non-fermenting colony suggested the presence of two organisms in intimate symbiosis difficult to separate by ordinary plating. The demonstration of similar agglutination properties in the case of the original and variant strains, the similarity of all cultural tests, with the exception of the one or two affected in the variation process, the fact that all the members of a species without exception may show the mutation when grown on a particular carbohydrate, *e.g.* all typhoid strains on iso-dulcitate agar, and the demonstration of the process in cases in which the culture was started from a single bacillus, disposed of this objection and established on a firm basis this mutation process as occurring in bacteria.

These facts being demonstrated, certain observers and thinkers have maintained that our methods of bacterial classification have been undermined thereby; they suggest that if these sudden changes affect one character of an organism it is not unlikely that they may affect several simultaneously, and the *B. typhosus* of to-day may have been some other organism yesterday and may be yet another to-morrow.

In this rather depressing chaos R. Müller (1909) has suggested that these mutations are in a very high degree specific. If it can be proved that each and every member of a particular species, in a particular environment, alters in the same way and in approximately the same time, and that no other species so vary, then the process which was supposed to undermine current methods of classification would rather associate itself with them and tend to amplify them. The evidence R. Müller has brought forward in support of the specific character of mutations is the following:

- (1) All the typhoid strains he examined (120) when plated on iso-dulcitate agar, produced papillated colonies. These papillae always appeared within five days.

- (2) Of 200 strains belonging to the coli-group, only three or four were able to produce these papillae when grown on the same agar. These few exceptions he compares to the organisms other than *B. typhosus* which agglutinate with a typhoid serum, of which a number have been described.

In view of the above facts he likens this iso-dulcitate mutation test to the agglutination test in point of delicacy for *B. typhosus*. These papillae produced by *B. typhosus* are found to contain a new typhoid strain which is unable, when grown again on iso-dulcitate media, to produce papillae.

R. Müller has shown that a similar raffinose mutation has a differentiating value as between *B. Aertryck* and *B. paratyphosus* B.

This proposition of R. Müller's seemed well worthy of investigation, and I therefore determined to select, from a series of organisms isolated from faeces and urine, those strains which had the power of "mutating" when grown on lactose-agar, and to examine the organisms so isolated to see if they formed one or more compact and well-defined species. For the sake of brevity I shall call such strains lactose-mutators, and similarly in the case of organisms showing mutation processes on raffinose- or saccharose-agar, I shall speak of them as raffinose- or saccharose-mutators respectively.

*The specificity of the lactose mutation in the case of
coli-typhoid organisms.*

The strains hereinafter to be described have been obtained mainly from the excretions of suspected typhoid carriers. The technique practised has been the following: the material was plated out on MacConkey's medium and, after 24 hours, a fair sample of the non-fermenting colonies was inoculated into mannite broth tubes. Those showing gas formation were not further examined in the search for *B. typhosus*. These mannite cultures formed the main source of my material and for them I am indebted to Dr Ledingham.

Relative frequency of lactose-mutators in faeces and urine.

Out of 50 successive samples of material which were plated on MacConkey's medium and incubated for 24 hours 16 showed lactose-fermenting colonies only. The other 34 samples produced colonies which at this interval of time appeared to be exclusively non-fermenting. Mannite cultures of 70 such colonies were examined by me. They were replated on MacConkey's medium a second time to exclude further the possibility of contamination, and care was taken to have scattered colonies so that fermentation could easily be detected. After 48 hours' growth 36 of these strains were found to be typical lactose fermenters, so that many of the original colonies from which they had been taken had been too small to show any acid reaction after the short space of

24 hours. The remaining 34 plates consisting of non-fermenting colonies only were incubated for seven days. It was found that during this period no less than 21 of them developed definite papillae on their colonies on these lactose plates. We must, therefore, look upon lactose-mutating organisms as really comparatively common in the gut, though Massini found only one strain in the material he examined. These 21 strains were now plated out again on lactose further to ensure purity in the material worked from; no contaminations were found.

Sub-cultures from papillated plate colonies must be carefully distinguished according to the portion of the colony from which they are taken. If they come from the flat portion of a colony they perpetuate the original race, if from the papillae they contain as a rule mixtures in varying proportions of the new and old strains. In addition to the 21 strains so examined, I am indebted to Dr Ledingham for two other strains isolated from faeces, and to Prof. McWeeney for one strain which was, I understand, obtained from faeces and which was agglutinated by a $\frac{1}{50}$ dilution of the serum of the patient from whom it was obtained. For appearances of these strains when plated out on MacConkey's medium, see Table I (pp. 200-2).

To sum up Table I, we find that there were 19 strains giving papillae with acid reaction within one week when grown at 37° C., three strains giving papillae in the same time, none of which were acid in reaction, and two strains giving no papillae until after one week's incubation.

This description depends on a naked-eye examination of the plates and is summarised as follows:

Papillae in seven days		Papillae later than seven days at 37° C.
Acid in reaction	Non-acid in reaction	
Strain 12	Strain 8	Strain 23
13	24	17
6	16	
18		
10		
19		
3		
2		
4		
21		
5		
14		
7		
15		
11		
20		
22		
9		
1		

Number of papillae per colony.

This varies greatly in different strains. It depends also on the length of time the plates have been incubated, and the size which the colony has attained. After four days the papillae may be non-existent in plates of some strains or as many as 40 on similar sized colonies of another strain. Strains 1, 9, 3, 2, 4 and 21 belong to a group showing 20 to 40 papillae at this stage, while the others show, excepting 24 where they are innumerable, diminishing numbers. Types are shown in the accompanying drawings (Plate I) and photographs (Plate II, figs. 1-4).

Size of papillae.

The papillae vary greatly in size in different strains. The absolute number of papillae has very little relationship to size. They may be few and small as *e.g.* in the case of strain 14, or very numerous and small as *e.g.* strain 24. The strains which show 20 to 40 papillae by the fourth day have papillae of moderate size. The largest papillae are got in the case of some of the strains showing few papillae on the colonies. These points are best illustrated by accompanying photographs (Plate II, figs. 1-4).

The strains were all plated out again on MacConkey's medium and incubated seven days at 37° C. and again they showed very similar appearances to those described in the foregoing Table I in respect of number of papillae, their reaction and time of their appearance. Plating was undertaken a third time from fresh single colonies of these strains. In this case the plates were incubated at 37° C. for five days and subsequently left at room temperature for a further 11 days. The appearances got in this series differed from the former only in the following particulars, viz. the acid reaction of the papillae was not present in the case of strains 10 and 19, while strain 17 which gave no papillae during seven days' incubation at 37° C. produced papillae on the plates grown 11 days at room temperature after five days' preliminary incubation.

On the nature of these strains.

There can be no doubt that the 19 strains which gave papillae of acid reaction on lactose-agar (see Table I) belong to the *coli mutabile* class, of which class Massini's organism was the first described member; two of the three strains, which produced in seven days naked-eye

TABLE I.

Cultures grown at 37° C. All observations naked eye unless otherwise stated.

The appearance of colonies of the following strains when grown on lactose-agar plates with neutral-red is summarized from one experiment as follows. None of these strains produced fermenting colonies, though the papillae they produced were in the case of the majority of strains of acid reaction.

Number and source of strain	After 24 hours	After 48 hours	After 72 hours	After 96 hours	After 7 days
1 Faeces	Smooth non-fermenting colonies only	As many as forty papillae on the largest colonies, many of the papillae are of acid reaction	...	... The central papillae of the colonies are more acid than those further from the centre. The extreme periphery shows no papillae in the case of this strain	—
2 " Ditto. (Plate II, fig. 2)	As many as twenty papillae were present on one colony. None acid in reaction	Up to forty papillae on some colonies	Ditto.	Papillae faintly acid in reaction	These three strains are and have been throughout very similar in appearance
3 " Ditto. ...				Papillae faintly acid in reaction	
4 " Ditto. (Plates I, and II, fig. 3)				Papillae faintly acid in reaction	
5 Urine	...	A few non-fermenting papillae present	The papillae reach as many as forty on some colonies As many as six non-fermenting papillae present on largest colonies	As many as eight papillae present on largest colonies. A few acid in reaction	—
6 Faeces	Ditto. (Plate II, fig. 1)	...	Well-marked naked-eye papillae present, none of acid reaction	As many as six papillae on one colony	As many as ten papillae present of slight acid reaction
7 " No papillae present. No fermenting colonies present	...	A few papillae present	As many as two papillae on one colony. The papillae have marked acid reaction	...	—

8	Faeces	Ditto.	...	No papillae present	Small papillae present	As many as six papillae present on single colonies, no acid reaction of papillae present	Papillae still show no acid reaction
9	"	Ditto.	...	As many as five papillae present on largest colonies. Some of the papillae are acid in reaction	...	As many as thirty-one papillae present on one colony	All signs of fermentation in papillae gone. They are now a dirty yellow colour
10	"	Ditto.	...	...	As many as four papillae on one colony. All non-fermenting	...	As many as ten papillae on colonies. A few of the papillae with faint acid reaction
11	Urine	Ditto.	...	...	As many as three non-fermenting papillae present	Non-fermenting papillae only	A few of the papillae very acid in reaction
12	Faeces	Smooth non-fermenting colonies only	...	All non-fermenting colonies with no papillae	...	Up to two large papillae on single colonies. The papillae are of acid reaction	Up to three intensely acid papillae on the largest single colonies
13	Urine	Ditto.	...	...	...	Up to three large papillae of acid reaction, on single colonies. Colonies all alkaline themselves	—
14	Faeces	Ditto.	...	...	...	No papillae present	A few small papillae present on colonies, one or two of the papillae are of acid reaction
15	"	Ditto.	...	No papillae present	As many as four colourless papillae of good size on the colonies	Non-fermenting papillae only	As many as six papillae on one colony, a few of the papillae are acid in reaction
16	Urine	Ditto.	...	...	No papillae present	A few non-fermenting papillae present	Many non-fermenting papillae present
17	Faeces	Ditto.	...	...	...	No papillae present	No papillae present after seven days' incubation. A few non-fermenting papillae appeared on the colonies after the plates had stood on the bench for some time later

TABLE I (Continued).

Number and sources of strain	After 24 hours	After 48 hours	After 72 hours	After 96 hours	After 7 days
18 Faeces	Smooth non-fermenting colonies only	...	...	No naked-eye papillae yet, few visible with a hand lens	As many as six papillae on one colony
19 Urine	Ditto. (Plate I, fig. 2)	...	...	A few single papillae present on some of the colonies	As many as seven papillae on one large colony, three of which papillae on one colony have definite acid reaction
20 "	Ditto.	...	...	Similar to strain 19 throughout	As many as four papillae on one colony. Some papillae intensely acid in reaction
21 Faeces	Ditto.	No papillae present yet	Papillae present, small, as many as six on large colonies	As many as twenty papillae present on largest colonies. The colonies look to-day very much like those of "2," "3," and "4" strains	Similar to strain 19
22 "	Ditto.	No papillae present	...	As many as seven papillae present on largest colonies. A few of the papillae are acid in reaction	—
23 "	Ditto.	...	...	...	Many small papillae present on examination with a hand-lens. No papillae present with naked eye. They appeared after being allowed to stand on the bench after seven days' incubation
24 "	Ditto. (Plate II, fig. 4)	...	...	No papillae present	Papillae very small and numerous. The colonies look like morocco leather on the surface. The papillae are not of acid reaction

papillae with no acid reaction, are, as will be subsequently shown, almost certainly of the same class.

Strain 23 which showed papillae of such small size that they required a hand lens for their observation, and strain 17 which only produced small neutral papillae when grown at room temperature for 11 days after previous incubation, connect this group with the non-lactose fermenters which do not show any tendency to throw off lactose-fermenting mutants. Strain "24" which formed tiny papillae but failed to ferment lactose after two months' growth on media containing it seems only a doubtful relative of this group.

Massini (1907) showed that if a papilla of his strain from a colony on a lactose plate be inoculated into ordinary broth and the latter plated on lactose-agar, a mixture of fermenting and non-fermenting colonies results. The non-fermenting colonies again produce papillae as in the case of the original strain, while the fermenting colonies give rise to a pure race of fermenting progeny which show no tendency to revert to the non-fermenting condition of the original strain. In my series of strains, replating on lactose-agar from papillae gave red and white colonies usually. The only negative strains were 15 and 24, where the replating gave only non-fermenting colonies; in the case of these two strains the replating from papillae was thrice repeated.

It must be remarked, however, that the proportion of red and white colonies on the plates is very variable when obtained in this way. The plates may sometimes consist of all fermenting or all non-fermenting colonies, in which case replating from a papilla has to be undertaken a second or even a third time, to get the typical mixed plates. The *permanency* of the fermenting character of these strains has not yet been tested, but our knowledge of other variants which arise suddenly in this fashion leads one to suppose that they will retain their fermenting character for many months, at least in those cases where the papillae appear early, *i.e.* from the second to the fourth day. Probably those arising later will have a slight tendency to reversion similar to dulcitate-fermenting variants of *B. typhosus* (Penfold, 1910).

The next question to consider was the behaviour of these strains when grown on litmus-lactose-peptone-water. The findings are recorded in Table II.

To sum up the results of Table II we may say that the strains take up the position of slow lactose fermenters except strain 24 which was entirely unable to ferment lactose. The seven strains showing partial acidity on the first day could only be called slow fermenters when

compared with the lactose-fermenting variants the same strain threw off, or when compared with normal strains of *B. coli*. There is not the slightest doubt that just as selection was seen to occur in the plates, so here it occurred in the broths, and the varying times taken to produce acidity doubtless depended on the number and the nature of the variants produced. It is interesting to note that all the strains showing a large number of papillae of acid reaction, excepting strain 21, gave in lactose-peptone-water relatively early acidity. Strain 6 gave only up to 10 papillae on its largest colonies and strain 7 only two in 72 hours, so that their early acidity in peptone water depended rather on the nature than the number of the variants. The variants were fewer but obviously better fermenters and could, therefore, take advantage of the lactose food supply very fully as soon as they arose.

Strain 24 after being grown 35 days on lactose-peptone-water was sub-cultured again with the same medium and again failed to produce acidity within 30 days. This strain as before stated seems to be a very doubtful relative of this group.

Sub-culture of the lactose broths of the slowly fermenting strains was practised, when it was found that the length of time taken to produce acidity in the second lactose-peptone-water culture was reduced to one to five days except in the cases of 23 which still took 14 days, and 24 which, as before stated, did not ferment the lactose-peptone-water at all. This subject will be dealt with when the variant strains are described.

The general biological properties of the strains are described in the Table III annexed. The carbohydrates were added to peptone water in a Durham's tube and the fermentation properties tested in the usual way.

By the normal strain is meant the strain as isolated from the body. The variant strain was in the majority of cases obtained by replating on lactose-agar a papilla from a neutral-red-lactose plate of the normal strain and selecting for sub-culture a red colony—the variant strain.

Where these plates did not show red colonies as in the case of strain 15, the lactose broth which had fermented was plated on lactose and readily yielded a fermenting colony. In the case of strain 24, however, even this method was not possible since as before stated it failed to ferment the lactose even in the peptone water tubes.

In Table III the differences between the normal and variant strains are alone noted, not the points of agreement.

The Ehrlich test for indol was used throughout.

The following abbreviations are used in this table to permit of concise statement:

Af = full acid reaction.

A followed by a fraction indicates varying degrees of partial acidity.

G = gas. Gn = no gas.

The fraction following the letter G indicates the volume of the gas tube filled by gas. Amounts of gas less than $\frac{1}{12}$ of the gas tube are indicated by p and p'; the former letter indicates a pinshead bubble, the latter a larger amount though less than $\frac{1}{12}$ of the tube. The gas tubes used were 2 inches long by $\frac{1}{4}$ inch internal diameter.

Further signs in the table are in common use and do not require explanation.

The following are the main facts to be culled from Table III:

A. The essential differences in cultural characters between the variant and original strains are, first, the original strain ferments lactose broth slowly; second, it does not clot milk or does so only slowly, while as a rule in the case of the new strains lactose broth is fermented and milk is clotted much more quickly.

B. Glucose, laevulose, mannose, galactose, arabinose, inulin, sorbite, and mannite are either fermented or not fermented as the case may be by all the members of the group examined, *i.e.* they afford no help in differentiating the members of the group from each other.

C. Strains 22 and 9 are the only two non-gas producers. This gives however no idea of the relative frequency in the *coli mutabile* class of gas-producing and non-gas-producing forms from faeces and urine, as 21 of my strains were selected from gas-producing organisms exclusively.

D. *Xylose* is fermented by all the members of the group. The variant strains of 1 and 9 do it more quickly than the normal strains, whereas in the case of 4 the opposite is the case.

E. *Iso-dulcitate*. "21" is the only strain not fermenting iso-dulcitate. Strains 12, 13, 6, 5, 18 and 14 all require 48 hours to ferment it, and strain 16 seven days. The other strains all ferment it in 24 hours. The gas yield from this sugar, in the case of the gas-forming strains, is always small. In this respect they agree with intestinal bacteria in general.

F. *Maltose*. All strains fermented maltose quickly, excepting "24," which did not attack it at all.

G. *Lactose*. The variant strains fermented this more quickly than the normal. Strain "24" did not attack it at all.

TABLE III. *General biological characters of the normal strains.*[illegible]

Date		Page	Page
1881	1882	1	1
1882	1883	2	2
1883	1884	3	3
1884	1885	4	4
1885	1886	5	5
1886	1887	6	6
1887	1888	7	7
1888	1889	8	8
1889	1890	9	9
1890	1891	10	10
1891	1892	11	11
1892	1893	12	12
1893	1894	13	13
1894	1895	14	14
1895	1896	15	15
1896	1897	16	16
1897	1898	17	17
1898	1899	18	18
1899	1900	19	19
1900	1901	20	20
1901	1902	21	21
1902	1903	22	22
1903	1904	23	23
1904	1905	24	24
1905	1906	25	25
1906	1907	26	26
1907	1908	27	27
1908	1909	28	28
1909	1910	29	29
1910	1911	30	30
1911	1912	31	31
1912	1913	32	32
1913	1914	33	33
1914	1915	34	34
1915	1916	35	35
1916	1917	36	36
1917	1918	37	37
1918	1919	38	38
1919	1920	39	39
1920	1921	40	40
1921	1922	41	41
1922	1923	42	42
1923	1924	43	43
1924	1925	44	44
1925	1926	45	45
1926	1927	46	46
1927	1928	47	47
1928	1929	48	48
1929	1930	49	49
1930	1931	50	50
1931	1932	51	51
1932	1933	52	52
1933	1934	53	53
1934	1935	54	54
1935	1936	55	55
1936	1937	56	56
1937	1938	57	57
1938	1939	58	58
1939	1940	59	59
1940	1941	60	60
1941	1942	61	61
1942	1943	62	62
1943	1944	63	63
1944	1945	64	64
1945	1946	65	65
1946	1947	66	66
1947	1948	67	67
1948	1949	68	68
1949	1950	69	69
1950	1951	70	70
1951	1952	71	71
1952	1953	72	72
1953	1954	73	73
1954	1955	74	74
1955	1956	75	75
1956	1957	76	76
1957	1958	77	77
1958	1959	78	78
1959	1960	79	79
1960	1961	80	80
1961	1962	81	81
1962	1963	82	82
1963	1964	83	83
1964	1965	84	84
1965	1966	85	85
1966	1967	86	86
1967	1968	87	87
1968	1969	88	88
1969	1970	89	89
1970	1971	90	90
1971	1972	91	91
1972	1973	92	92
1973	1974	93	93
1974	1975	94	94
1975	1976	95	95
1976	1977	96	96
1977	1978	97	97
1978	1979	98	98
1979	1980	99	99
1980	1981	100	100

H. *Raffinose*. Nine of the strains fermented this sugar, viz. 16, 9, 12, 13, 5, 14, 21, 24 and 15.

I. *Saccharose*. Twelve strains were positive on this sugar.

J. *Dextrin*. All strains fermented this except "24."

K. *Salicin*. Nine strains fermented this glucoside, three in 48 hours, and the rest within seven days.

L. *Glycerine*. All strains fermented this; the time varied from one day to one week.

M. *Adonite*. Strains 7 and 10 were the only ones to ferment this.

N. *Dulcite*. Eight strains were negative, the rest positive.

O. *Sodium formate*. All strains produced gas from this salt except "22" and "9" which failed to produce gas from any of the carbohydrates.

P. None liquefied *gelatine*.

Q. All acidified *milk*, the variant strains more intensely than the normal.

R. *Indol* production occurred in the case of 20 strains.

TABLE IV.

No. of strains	Iso-dulcite	Saccharose	Raffinose	Salicin	Adonite	Dulcite	Motility in 24 hours broth	Gas formation	Indol
6	+	—	—	—	—	+	+	+	+
18	"	"	"	"	"	"	"	"	"
4	+	—	—	+	—	+	—	+	+
3	"	"	"	"	"	"	"	"	"
2	"	"	"	"	"	"	"	"	"
14	+	+	+	—	—	+	—	+	+
12	"	"	"	"	"	"	"	"	"
5	"	"	"	"	"	"	"	"	"
13	"	"	"	"	"	"	"	"	"
15	"	"	"	"	"	"	"	"	"
1	+	—	—	+	—	—	+	+	+
23	"	"	"	"	"	"	"	"	"
10	+	—	—	—	+	—	—	+	+
21	—	+	+	+	—	—	—	+	—
7	+	—	—	+	+	—	+	+	+
11	+	—	—	—	—	—	—	+	—
19	+	+	—	—	—	+	+	+	+
8	+	—	—	+	—	+	+	+	—
24	+	+	+	—	—	+	+	+	+
17	+	+	—	—	—	—	+	+	+
16	+	+	+	—	—	+	—	+	+
22	+	+	—	—	—	+	—	—	+
9	+	—	+	—	—	—	+	—	+
20	+	+	—	+	—	+	+	+	—

Staining and Morphology. All were gram-negative rods. The majority (13) were non-motile in 24 hours broth culture. Voges and Proskauer's test was in all cases negative when carried out on 48-hour cultures.

Many of the tests used are seen to have no differentiating value in the series of organisms examined. The preceding table gives briefly those that have value (Table IV).

This table shows that the 24 strains comprise no less than 16 varieties. Amygdalin which was fully fermented by strain "16" produced in other instances various shades of green. I have omitted it from the table of classification, as strain "16" was already differentiated.

The scheme of classification of these forms mutating on lactose is a little different from that used by MacConkey (1909) for ordinary forms of *B. coli*. His recommendation was followed in not using the reduction of nitrates or litmus-whey tests. These he found of little differentiating value. Further I did not use inosite. On the other hand, iso-dulcite, raffinose, and salicin were found of distinct differentiating value and they are therefore included in the synoptic table.

The motility tests were made with 24-hour broth cultures and this may account for the relative frequency of non-motile organisms. MacConkey's tests for motility were done in 6-hour broth cultures which gives a greater proportion of positive results. Leaving aside the tests wherein Table VI differs from MacConkey's scheme and comparing his table with mine in respect of the identical tests we find:

MacConkey's examination of 178 strains of lactose-fermenters from human faeces showed three large groups, viz.

- | | | |
|-----|---|--|
| 1st | { | <i>B. Grünthal</i> and its non-motile form. |
| | { | <i>B. vesiculosus</i> . |
| 2nd | { | <i>B. coli communis</i> and its non-motile form. |
| | { | <i>B. Schöfferi</i> . |
| 3rd | { | <i>B. 71</i> and its non-motile form. |
| | { | <i>B. neapolitanus</i> . |

Group 1 had 41 out of the 178 strains.

Group 2 had 48 out of the 178 strains.

Group 3 had 57 out of the 178 strains.

Out of the 24 strains of my series

2 belonged to Group 1.

6 belonged to Group 2.

8 belonged to Group 3.

Therefore my largest groups correspond fairly to those of MacConkey's table.

The three groups in question constitute 82% of MacConkey's series and 66% in my series, and if the strains of urinary origin be excluded from my series a still closer approximation to MacConkey's series is obtained. That is to say, these organisms of the mutabile group, unable to ferment lactose without variation and selection, show a very similar constitution judged by cultural characters to the lactose-fermenters of the faeces. It appears not at all improbable from this consideration that the *coli mutabile* organisms probably give rise to coliform organisms by variation and selection within the gut, and that the ultimate flora of the gut depends largely on the nature of the non-lactose fermenting strains which get into it and are able to throw off lactose-fermenting variants.

It is noteworthy that strain 6 after the process of variation and selection produced a typical *B. coli* Escherich strain in the strictest use of the term. The *B. coli mutabile* of Massini did not produce indol. I believe no genuine *B. coli* Escherich in the strict use of the term has been previously experimentally selected.

Little has been done with these strains in respect of agglutination tests, as they have been found to give little help in classifying the coliform organisms of the gut. I made, however, a serum from a typical *B. coli* Escherich and tested it against strains 4, 3 and 2 with the following result:

	1/50	1/250	1/1000	1/5000
4	...	—	—	—
3	—	—	—	—
2	?	—	—	—
Normal <i>B. coli</i>	+++	+++	+++	++

Further strains were not tested.

The specificity of the lactose mutation.

It would appear therefore that a large number of organisms exist in the gut which are able to throw off lactose-fermenting mutants. These strains appear to belong to many species which are closely related to the lactose-fermenting species found in the same situation. The mutation process as it occurs on lactose media is therefore not specific in any strict sense.

Of course it might be maintained that the different varieties of lactose-fermenters found in the intestine are not separate species. There seems no inherent reason for believing that fermentation tests

have one value when applied to the non-lactose fermenters and another when applied to the lactose-fermenters. If *B. typhosus* is a distinct species as opposed, for example, to the dysentery organisms, then one must admit that *B. lactis aerogenes* and *B. coli* Escherich are equally good species though they may not produce such characteristic pathological conditions.

The specificity of the iso-dulcitate mutation.

Reiner Müller showed that all natural strains of *B. typhosus* which he examined threw off mutants on iso-dulcitate media. I examined a large number of strains and confirmed this. He further showed that a few pseudo-dysentery strains do this, but out of about 200 coliform organisms examined, only three or four possessed this property.

If this latter assertion is supported by further work, then the mutation test must be admitted to have a high degree of specificity in this case.

R. Müller has further stated that the same strain will only produce papillae as a rule on one carbohydrate. He states that *B. typhosus* did not show this mutation on any other of 18 carbohydrates tested, one of these being dulcitate. This latter statement is, however, not correct as I have elsewhere shown. It seems probable that the same organisms may vary suddenly in several directions.

The raffinose mutation.

R. Müller found that *B. paratyphosus* B. threw off mutants as shown by papillae-formation, when grown on raffinose agar, while *B. Aertryck* did not do so. This appears to be the only cultural distinction between the two varieties.

I am indebted to Dr F. A. Bainbridge for 29 strains of the food-poisoning group which I classified according to this test into two groups, viz. (1) *B. paratyphosus* B. and (2) *B. Aertryck*.

The strains were plated on raffinose agar and observed daily. Significant differences between the colonies of the different strains did not appear until the 5th day and they became more marked by the 7th and 9th days. The intensity of papillae-formation, and time of appearance of the same, are indicated in the following Table V. None of the strains showed papillae of acid reaction though neutral red was present in the plates.

The points to be noted in the table are :

(1) The four strains of *B. enteritidis* Gaertner produced in three cases papillated colonies, being in this respect like *B. paratyphosus* B.

The test is, therefore, not strictly specific for *B. paratyphosus* B.

(2) All the pure *B. paratyphosus* B. strains produced papillae except "*Murray, original*"; the number and size of the papillae were, however, very various in the case of the different strains and also of the same strain at different times.

(3) One strain only of *B. Aertryck* showed papillae, viz. "*S. P. Wassermann*" and in this case they were of great number and large size; strain "*Victoria*" showed one papillated colony only, on a crowded plate. See illustrative photographs (Plate III, figs. 5, 6, 7).

A mixed group of 10 of the above strains were plated out again on raffinose agar, and all gave the same result except that strain "*M. 5*" showed 20% of its colonies papillated.

Conclusions :

(1) Theoretical. The raffinose mutation is not strictly specific for *B. paratyphosus* B. as *B. enteritidis* Gaertner may show it, and occasional strains of *B. Aertryck* also.

(2) The raffinose mutation is however the only cultural means of helping to distinguish between *B. paratyphosus* B. and *B. Aertryck*. It will but rarely err in separating these two varieties and, having regard to the difficulty and occasional impossibility of the absorption agglutination test, it has a distinct practical value.

Interconversion of B. Aertryck and B. paratyphosus B. in respect of the mutation test.

"*M. 5*" which showed up to 20% of papillated colonies and "*Victoria*" which showed only one colony with papillae were sub-cultured on to agar from the smooth interpapillary area of papillated colonies, and in this way pure strains were obtained, all the colonies of which produced papillae. Similarly strains of *B. paratyphosus* B. had sub-cultures taken from the papillae, and these on plating readily yielded strains which were unable to produce papillae.

Dr Bainbridge, who intended to see if alteration of the mutation test in the case of these strains was accompanied by alteration of the absorption agglutination tests, unfortunately never had the opportunity to carry this out.

TABLE V.

To determine the value of the raffinose mutation in separating *B. paratyphosus B.* from *B. Aertryck*.

The following 29 strains, all being members of the food-poisoning group, were obtained from Dr Bainbridge in order to divide them into two groups, viz. *B. paratyphosus B.* and *B. Aertryck*. After having classified them the results were compared with Dr Bainbridge's results obtained by the Castellani absorption test.

Name of strain	Condition of raffinose plate on the 5th, 7th and 9th day of incubation				Intensity of papillae formation	Source of strain	Classification according to Castellani's method by Dr Bainbridge	Remarks
	5th day of incubation	7th day of incubation	9th day of incubation					
Trautmann 5	Definite papillae seen with a hand lens	As last observed	...	A few naked-eye papillae present	+	Healthy man	<i>B. paratyphosus B.</i>	—
Danyz R	" " "	Small naked-eye papillae present	" "	As last observed	+	Stock strain	<i>B. enteritidis</i> of Gaertner	—
Potter	" " "	" " "	" "	Many papillae of moderate size present on all the larger colonies	++	From bone abscess after paratyphoid fever	<i>B. paratyphosus B.</i>	—
Danyz H	" " "	Papillae only visible with a hand lens	" "	A few naked-eye papillae seen on all the larger colonies	+	Stock strain	<i>B. enteritidis</i> of Gaertner	—
Prigge 9	Many naked-eye papillae present	As last observed	...	Up to 40 or 50 papillae present on larger colonies with naked eye	++	Carrier case	<i>B. paratyphosus B.</i>	—
Prigge 5	A few naked-eye papillae present	Many definite naked-eye papillae	" "	Up to 30 naked-eye papillae present on single colonies	++	" "	" "	—
Savage W	Many large naked-eye papillae present	" " "	" "	Every colony covered with large well-marked papillae	++	From case of paratyphoid fever	" "	Plated frequently on the medium and always found papillate to the same intense degree
MacWeeny	" " "	" " "	" "	60% of the colonies show numerous and large papillae	++	" "	" "	—
Trautmann 2	No papillae present	No papillae	...	No papillae	0	From food	<i>B. Aertryck</i>	...

Murrow	...	"	"	"	"	"	"	0	From case of food poisoning	"	"	—
Eichou	...	"	"	"	"	"	"	0	"	"	"	—
W. 9. B.	...	"	"	"	"	"	"	0	From food ...	<i>B. enteritidis</i> of Gaertner	...	—
Trautmann I	"	"	"	"	"	"	"	0	From case of food poisoning	<i>B. Aertryck</i>	...	—
Trautmann 3	"	"	"	"	"	"	"	0	"	"	"	—
M. 5	...	"	"	"	"	"	"	0	Stock strain	"	...	—
840	...	"	"	"	"	"	"	0	From healthy pig	"	...	—
Prigge 6	...	"	"	"	Many definite naked-eye papillae present	Many papillae	...	+	From healthy man	<i>B. paratyphosus</i> B.	...	—
Victoria	...	"	"	"	No papillae	No papillae except on one colony	...	0	Fatal food poisoning case	<i>B. Aertryck</i>	...	—
McAlister	...	No naked-eye papillae present	No naked-eye papillae	"	"	A few papillae got on a few colonies. They are small in size	...	+	From water	<i>B. paratyphosus</i> B.	...	—
Fowler 3	...	"	"	"	Many naked-eye papillae on all the colonies	As last observed	...	+	From case of paratyphoid fever	"	"	—
Savage	...	"	"	"	Many large naked-eye papillae on all the colonies	"	...	+	"	"	"	—
Prigge 4	...	"	"	"	No papillae present	No papillae	...	0	Healthy man	<i>B. Aertryck</i>	...	—
Murray Original	"	"	"	"	"	"	...	0	Doubtful case	<i>B. paratyphosus</i> B.	...	—
W	...	"	"	"	Papillae formation well marked	As last observed	...	+	<i>B. typhi murinae</i>	<i>B. enteritidis</i> of Gaertner	...	—
S. P. Wassermann	"	"	"	"	"	"	...	+	Unknown	<i>B. Aertryck</i>	...	—
Prigge 1	...	"	"	"	"	"	...	+	Case of paratyphoid fever	<i>B. paratyphosus</i> B.	...	—
Fowler 1	...	"	"	"	"	"	...	0	Food poisoning	<i>B. Aertryck</i>	...	—
O'Brien	...	"	"	"	"	"	...	0	Guinea pig epizootic	"	"	—
Rommeler liver	"	"	"	"	"	"	...	0	Healthy liver	"	"	—

Saccharose results.

There is not yet sufficient information forthcoming to enable us to determine to what extent the saccharose mutation of intestinal bacteria is specific for them, but six strains of saccharose mutating bacteria have been very shortly described by Thaysen (1911) and with the few tests he described he could classify them into two groups, so that preliminary observations suggest that the saccharose mutation is not very specific.

B. typhosus and dulcitate.

B. typhosus has been shown by the author to produce papillae on dulcitate agar. Some strains produce the papillae early, in which case they are of acid reaction if a suitable indicator is present in the agar.

The process here is, however, not a mutation in the strictest sense, since the fermenting power so acquired tends to disappear unless the growth on dulcitate media is continued for two or three months.

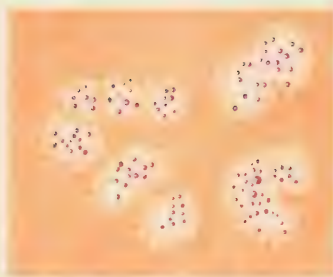
All typhoid strains agree in being able to throw off dulcitate-fermenting variants readily. Of one batch of 14 strains tested by the writer all of them produced acidity within four days in the second sub-culture tubes of litmus-dulcitate-peptone water in which they were grown. Many other strains were tested since and have behaved in the same way.

It may be quite definitely accepted that the ready power to throw off dulcitate-fermenting variants is a character of the *B. typhosus*. This character has been shown, however, to belong to certain water bacteria (Meyers Coplan 1909) and to Morgan's No. 1 *Bacillus* (Twort (personal)), so that while it is possessed by all typhoid strains it is deprived of much of its specific value by being common to some other bacteria.

The specificity of the chloracetic acid mutation.

The addition of sodium monochloracetate to agar medium has been shown (Penfold 1911) to cause many intestinal organisms to form papillae on their colonies (see photographs 8 and 9); these papillae are more highly resistant to chloracetate of soda than the original strain. This sudden acquirement of resistance to the medium may be reasonably looked upon as a mutation, but unlike the mutations previously dealt with, the strain is still able to produce papillae on the same medium in the presence of higher concentrations of the salt.

B. coli Escherich, *B. enteritidis* Gaertner, *B. paratyphosus* B., *B. paratyphosus* A., *B. Grünthal* and many other organisms have papillae-forming



A lactose mutating strain grown six days on neutral-red lactose-agar plate.
Strain IV in Table I. Papillae numerous.



Colonies of strain 19 on a neutral-red lactose-agar plate, grown
nine days at 37° C. See Table I. Papillae few.





Fig. 1. *B. coli mutabile*, strain "6";
plate seven days old.

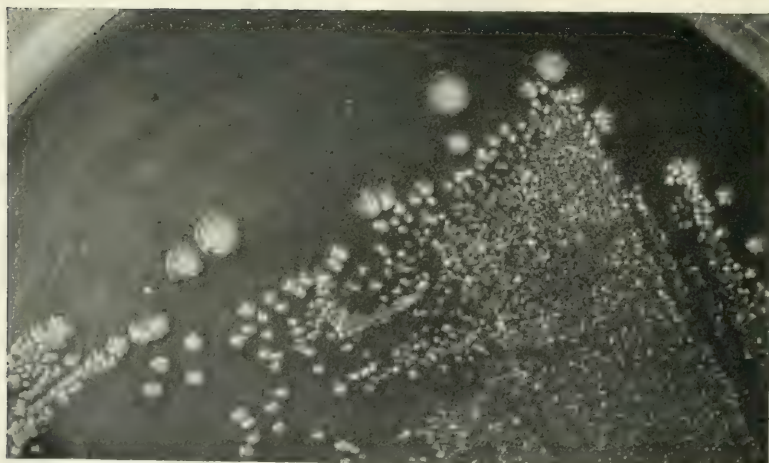


Fig. 2. *B. coli mutabile*, strain "2";
plate three days old.

PLATE II shows photographs of lactose agar plates of four of the strains described in the paper as lactose mutators. All the plates were incubated at 37° C. during the time indicated.



Fig. 3. *B. coli mutabile*, strain "4";
plate two days old.



Fig. 4. *B. coli mutabile*, strain "24";
plate seven days old.



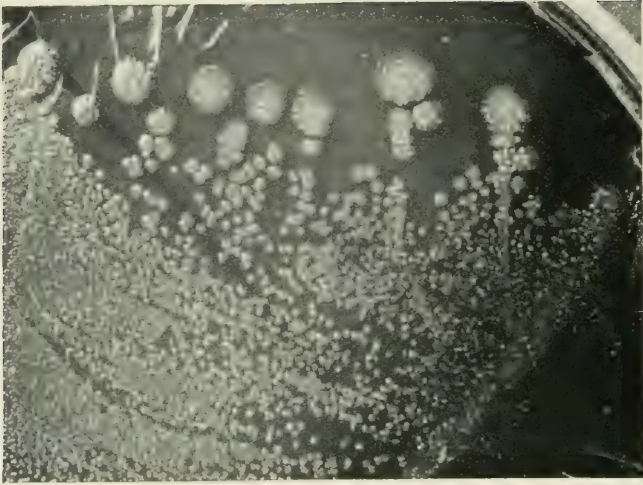


Fig. 5. *B. paratyphosus* B. grown for nine days on raffinose agar at 37° C.
A typical strain. Observe characteristic papillae.

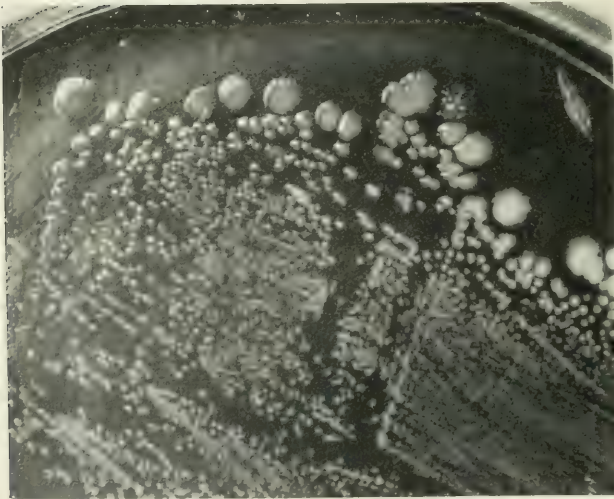


Fig. 6. *B. suispestifer*, strain "Victoria" grown on raffinose agar. The strain appears atypical since one of its colonies is shown to be papillated.



Fig. 7. *B. suispestifer*; showed typical smooth colonies only on raffinose agar though the plate had been incubated nine days.

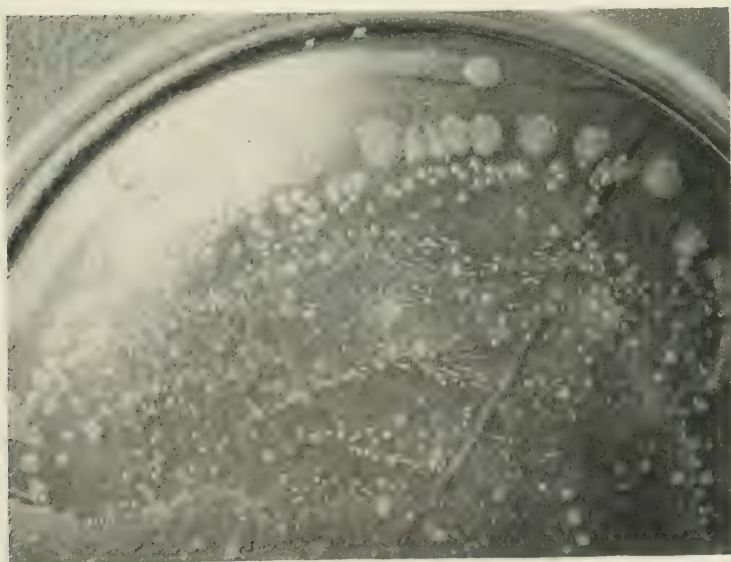


Fig. 8. *B. coli* Escherich after seven days growth on chloracetic acid agar.



Fig. 9. *B. coli* Escherich on chloracetic acid agar after twelve days growth. One large colony magnified about four times and presenting an enormous number of papillae.

power on this medium, but none of the typhoid strains (nine) examined possessed it.

The degree of specificity in this instance is apparently small.

Inability to vary quickly in particular directions is probably just as characteristic of the species as ability to vary. It took Twort two years to produce by constant growth on lactose a lactose-fermenting variant of *B. typhosus*. I tried rapid sub-culture on lactose-peptone water of a number of strains of *B. typhosus*, carrying on the process as far as the fiftieth generation in some cases, without obtaining the slightest sign of a new strain. I have also tried monthly and fortnightly sub-cultures with a large number of strains, and found that 18 months' growth on lactose failed to make any of them lactose-fermenters. We may therefore state that *B. typhosus* as a species is unable to produce readily lactose-fermenting variants and any organism readily taking on the power to ferment lactose is not *B. typhosus*. As previously mentioned not one of the eight strains of *B. typhosus* grown on chloracetic agar could produce papillae.

Similarly I have tried to obtain a saccharose-fermenting variant from a *B. coli* Escherich strain, but in spite of sub-culture on saccharose-peptone water twice weekly for over seven months, I found such a variant did not arise.

Eight months' growth of *B. enteritidis* Gaertner on milk was tried in the case of six separate series with the hope of removing or diminishing the preliminary period of acidity by which the organism is characterized when grown on milk; no change, however, occurred in this or any other property of the organism.

Single cell culture (Burri's method).

This method has been applied several times by me to strains of *B. coli mutabile* and I have readily succeeded in getting pure cultures from single cells. These cultures grown on lactose throw off the lactose-fermenting variants in exactly the same way as strains which have been obtained by the ordinary plate method.

GENERAL CONCLUSIONS.

The greatest degree of specificity of which we can conceive is that a particular property shall belong to every member of a species and shall belong to no other species or individual strain of such species. This degree of specificity is not, however, known to be shown by any

bacterial variation process. The nearest approach to it is the *iso-dulcité* mutation of the *B. typhosus* we have before mentioned, which fails, however, in so far as a very few other organisms have been found which show the same adaptability to this medium.

Nearly all strains of *B. paratyphosus* B. mutate on *raffinose* agar, but *B. enteritidis* Gaertner may do so and rarely the *B. Aertryck* (or *B. suipestifer*).

In respect of the *lactose* mutation it appears to be a common property of many bacterial species and has practically no specific differentiating value at all.

It is yet premature to say whether every member of any species possesses this property of mutating when grown on lactose.

The power to vary quickly in respect of *dulcité* appears to belong to every strain of *B. typhosus*.

The inability to vary quickly in respect of certain carbohydrates would appear to be as characteristic of certain species as the power to vary.

It will be noticed that the above examples of varying degrees of specificity of variation processes are taken entirely from the intestinal group of organisms. They are, therefore, not at variance with, though very distinct from, the haphazard variations that have been described in the case of the streptococci.

In sending this paper to the press I desire to thank Dr Ledingham for much helpful criticism in the course of this research, Dr Reid of Ealing for the excellent photographs he took of the illustrative plates and Miss Rhodes for the coloured drawings.

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An Attempt to differentiate the Diphtheroid Group of Organisms.

By HENRY PRIESTLEY.¹

THE true Klebs-Loeffler bacillus of diphtheria is, in its typical form, a definitely banded slender rod with distinct granulations, and when it occurs in this typical form is usually readily enough recognized by its morphology alone. But this is not the only, nor indeed, perhaps, the commonest form of the diphtheria bacillus. Great variations occur in the length and thickness, the prominence and number of the bands and in the distinctness of the polar granules, and it is occasionally very difficult, if not impossible, to recognize the diphtheria bacillus under these altered morphological conditions. This difficulty is accentuated and made more important by the fact that there is a large group of organisms which more or less closely resemble the diphtheria bacillus, but which do not possess a like significance from the bacteriological or public health point of view. Fortunately these diphtheria-like bacilli have their most frequent habitats in regions other than the throat. This group of diphtheroid or diphtheria-like bacilli thus attains an artificial importance, from the possibility of mistaking its members for the true diphtheria bacillus, quite apart from any pathogenic effect they may have on their own account.

While many examples of the group have been described in the literature, our knowledge of them is in a very unsatisfactory state and no really serious attempt has been made, on the one hand, to classify them among themselves, and, on the other, to furnish some readily applicable test to distinguish them from the true diphtheria bacillus.

The present work was undertaken to determine whether the present bacteriological methods were sufficient to supply such classification and distinction. In the event of these proving unsatisfactory it was hoped that some further methods might be devised to attain these

¹ From the Lister Institute, London.

ends, but owing to illness and other causes this part of the work had to be abandoned. It has been deemed advisable, however, to publish the experimental results thus far obtained, although the conclusions reached are largely of a negative character.

BRIEF REVIEW OF THE BIBLIOGRAPHY ON THE DIPHTHEROID ORGANISMS.¹

Diphtheroid organisms—i.e., organisms resembling more or less closely the diphtheria bacillus—have been isolated from almost every part of the human body. They have been found in the nose and throat in normal and pathological states of these parts [1 to 13], in the ear [3, 7, 14 to 27], conjunctiva [3, 28 to 30], urine and urinary tract [31 to 41], the female genital tract [31, 33, 36, 42], in the pus of a liver abscess [43], in empyema [44], in the vegetations of ulcerative endocarditis [45], in the skin in various conditions [24, 30, 31, 46 to 48], in association with leprosy in man [49 to 55], and the so-called rat leprosy [56], and also in the papules of variola and vaccines [30, 57 to 64], and in cellulitis [98]. Much has been made, also, of their occurrence in the cerebrospinal fluid [65 to 68]. Finally they have been observed in milk [31, 60, 69 to 71], in water [72], and in various animals [7, 39, 73, 74]. The most familiar of the diphtheroid organisms is the *Bacillus Hofmanni*, and as it has been described by very many workers, and is generally held to be a definite organism with stable characters, it has not been considered here, and the same is the case with the *Bacillus xerosis*.

Other varieties of diphtheroids have been described in more or less detail by their discoverers, but sufficient data have not been given in most cases to enable one to bring them together into groups, or indeed, in many cases, to say whether an organism has been described before or not. The only attempt which has been made to classify the members of the group from published descriptions is that by Graham Smith [39].

Some writers [18, 20, 78] have laid stress on the association of diphtheroids with the otitis media following scarlet fever, and in the series described below will be found twenty-five strains isolated from cases of scarlatina. Ford Robertson and his co-workers [32, 33, 66, 67] believe that diphtheroids are the causal agents of general paralysis

¹ As the bibliography of the subject is an extensive one, it has been found convenient, for reasons of space, to employ reference numbers in the text instead of authors' names.

of the insane and of locomotor ataxia, but the consensus of opinion is against this view [75], although some authorities believe that they may have some effect in the production of the terminal symptoms of these diseases [68]. The only other diphtheroids which have been claimed definitely as causal agents of disease in man are those found in sore throats by Ruediger and others [4, 5, 6], the *Bacillus coryzæ segmentosus* in "colds" [76], and the acne bacillus of Sabouraud.

In other cases the association of diphtheroids with morbid conditions has not usually been strongly insisted upon and their importance for human pathology has not been considered great.

SOURCE OF STRAINS EXAMINED IN THE PRESENT RESEARCH.

For this investigation diphtheroids were obtained from the mouth, nose, eye, ear, urine, urethra, vagina, and vulva, pus from tuberculous abscess of the hip, the skin in leprosy, and a few from unknown sources. Forty-nine strains in all were examined, of which twenty-five came from ears and the results are tabulated in the accompanying table. The preponderance of strains from ears was intentional.

It was hoped that some definite type might be found around which to group the remaining types. Moreover, many authorities [17, 19, 21, 22, 77] have described the occurrence of diphtheria bacilli in the ears of scarlet fever patients usually after very imperfect examinations, and it was hoped that this matter might be cleared up. Further, several authorities [18, 20, 78] have regarded the otitis media following scarlet fever as due to the presence of diphtheroids, and it was therefore thought that some additional light might be thrown on this question. On this last point, however, no conclusions were arrived at.

Strains from Otitis Media following Scarlatina.

Although sixty-nine cases of scarlatina were examined, and I am very much indebted to Dr. Thompson, of the North-Eastern Fever Hospital, for material, only four of these had otitis media. From three of these four cases strains of diphtheroids were isolated. These were identical in all the characters investigated (*see* Table S5) and correspond to no other strain isolated. It may be remarked that all three cases were in the same hospital at the same time, so that this fact, with the small number of observations, precludes the possibility of any conclusions being drawn. From the fourth case no diphtheroid was isolated.

Strains from the Ear.

In the examination of the ears the procedure was in all cases the same. Swabs were taken in the usual way, and cultures made on solidified serum. After twenty-four hours' incubation, and, again, after a further twenty-four hours' incubation, the growth, if any, was examined, and if diphtheroids were observed in smears stained with Loeffler's methylene blue, plates were made with serum agar, and the diphtheroids isolated where possible. The ears of eighteen normal persons were examined in this way, and in fifteen of them diphtheroids were observed. Of these, eight were isolated and examined in detail (*see* Table, E). These eight strains were identical, and the remaining seven, which were not isolated in the pure state, were morphologically, to all appearances, the same organism. They correspond in all details, so far as data are available, with the diphtheroid *Bacillus ceruminis* isolated by Graham Smith [7] from normal ears.

It seems reasonable to infer, therefore, that this is the type most commonly met with in normal ears, and hence its finding in pathological states must be considered to have less significance than it might otherwise have.

Strains from the Ear in Scarlatina.

From the sixty-nine cases of scarlet fever diphtheroids were obtained in thirty-two. Of these, twenty-five were isolated and examined in more or less detail. They showed, however, little uniformity, and fall into as many as nine groups. One group corresponds to the strain met with in normal ears, and consists of only two members. Another group (S5) of three members was found in the otitis cases. The largest group (S13) is represented by four members, and another strain closely resembling, or identical with, *Bacillus Hofmanni* contains three members (S1). No other strain consists of more than two members, and the majority contain only one.

Hence it appears that there is no special type of diphtheroid occurring in the ears in scarlatina, and they are not more common than in normal ears. Of course, the differentiation depends on the value which can be placed on the methods used, of which more will be said later; but some of them differ very markedly from one another—e.g., S13 and S38. A similar lack of uniformity was found in strains examined from other sources than ears.

Strains from other Sources.

I am indebted to Dr. Eyre, of Guy's Hospital, for many cultures from various sources containing diphtheroids, and to Dr. Arkwright and Dr. Bayon for pure cultures of C16 and B respectively. The remaining material was obtained from the Diagnosis Department of the Lister Institute.

The sixteen strains from sources other than ears fall into thirteen groups, most of which consist of only one member. One group isolated from urethral pus in cases of gonorrhœa consists of three members.

Hence it appears that the diphtheroids which are met with in various parts of the body form a very large series which cannot be arranged in groups, and vary as much among themselves as the equally ubiquitous streptococci.

The differentiation of the diphtheroids, as a whole, from the true Klebs-Loeffler bacillus is usually easy enough, if one takes into consideration all the cultural and morphological characteristics. There is no one character, however, which can be looked upon as reliable.

As we have seen, in the case of some strains of diphtheroids, the morphology is of little assistance. The fermentation reactions do not afford a much more reliable guide, except as regards *Bacillus Hofmanni*. These have been investigated to some extent by several workers [79 to 83].

The usually accepted fermentation reactions of the *Bacillus diphtheriæ* have been placed in the table for convenience of reference. It will be seen that in several cases the fermentation reactions of several diphtheroids (S19, G13, G5), which were undoubtedly not *Bacillus diphtheriæ*, approximate very closely to those of *Bacillus diphtheriæ*, especially when one considers that the acid production from saccharose and lactose is not usual in *Bacillus diphtheriæ*, and from glycerine and dextrine, is not invariable.

The formation of acid from dextrose has been frequently looked upon as being considerably in favour of an organism being *Bacillus diphtheriæ*; but it will be seen from the table that it has proved unreliable in differentiating the strains I have examined. Of the eighteen strains tested, fifteen produced acid from this sugar, and six of them (G13, C16, G12, D1, X, and S5) within twenty-four hours.

Myer Coplans [92, 93] makes use of a medium consisting of serum, glucose, neutral red, CaCl_2 and KCNS. "Colonies of diphtheria

appear almost invariably to yield a bluish-pink tint with diffusion of like tint through the medium; but with *Bacillus Hofmanni* the growth is yellowish and alkaline." This medium was tried in several cases, and found to be quite satisfactory in distinguishing between a typical acid-producing diphtheria bacillus and a non-acid-producing bacillus like *Bacillus Hofmanni*. Goodman [94] and Clark [95], however, have shown that different strains of the diphtheria bacillus vary considerably in acid production from glucose, from almost nothing up to a large amount; so that for the low acid producers this medium would not be satisfactory. Then, too, it will not serve to distinguish between the diphtheria bacillus and acid-producing diphtheroids.

PATHOGENICITY EXPERIMENTS.

None of the strains tested was found to kill a guinea-pig of 250 gm. when 2.5 c.c. of a forty-eight-hour broth culture was injected under the skin of the abdomen.

The usual method of injecting a given quantity of a twenty-four or forty-eight-hour broth culture into a guinea-pig is not satisfactory for comparative purposes, as even in the case of two strains of the same organism—say *Bacillus diphtheriæ*—1 c.c. of one culture may contain many millions more organisms than 1 c.c. of another culture.

Experiments with the object of devising a more satisfactory method of giving more uniform doses were in progress, but had to be abandoned. Arkwright¹ has recently recorded experimental results dealing with the variations in virulence of *Bacillus diphtheriæ*.

SEROLOGICAL TESTS.

Immunity reactions have been used by various authorities in the hope of differentiating the *Bacillus diphtheriæ* from some of the diphtheroids [84 to 89]. These were investigated to some extent, and found to be unsatisfactory.

AGGLUTINATION EXPERIMENTS.

A rabbit was immunized against *Bacillus diphtheriæ* by nine injections, first of killed bacilli, and later of living bacilli up to two agar slopes of living virulent bacilli. The serum from this animal was found

¹ *Journ. of Hyg.*, xi, p. 409.

to agglutinate the homologous strain completely in a dilution of 1—1,600, and had no effect on *Bacillus Hofmanni* and the diphtheroid tested (D1)

The great majority of genuine diphtheria strains tested (twelve out of fifteen strains) agglutinated spontaneously, however, so that the method cannot be considered a practicable one. Various means were tried to prevent this spontaneous agglutination, such as the addition of glycerine, formalin, various salts, &c., but with no success.

The agglutination of the homologous strain, moreover, presented some curious features. There was little or no agglutination after three hours at 37° C., and it was not marked until a further period of twenty-four hours had elapsed at room temperature, after which it proceeded rapidly and was almost complete at 1—1,600 in a few hours, the controls remaining throughout free from agglutination. It was found that the use of a solution containing 1 per cent. of KCl and 0·25 per cent. of CaCl₂ instead of normal saline solution, for dilution purposes, accelerated the action markedly, for there was distinct agglutination in six hours. Moreover, this solution increased the action, for agglutination was complete at 1—6,400 instead of 1—1,600, the controls remaining unchanged.

PRECIPITATION EXPERIMENTS.

Precipitation experiments were also tried with this serum, using as antigen an extract prepared from bacilli by Dean's method [90].

A precipitate at 1—20 was obtained with the homologous bacillus, but not with several other strains of *Bacillus diphtheriæ* tested. Later on an extract was prepared by Rowland's method [91] and gave a precipitate at 1—32, but this could not be followed up.

COMPLEMENT FIXATION TESTS.

These gave absolutely no results, probably owing to the want of a satisfactory antigen.

Bacterial extracts prepared by Dean's methods, and bacterial solutions in weak antiformin, were used without success. It will be seen that the immunity reactions examined were without exception unsatisfactory, and a further investigation seemed necessary before applying any of the reactions to the diphtheroid organisms, but illness prevented the carrying out of this.

	Source	Morphology	Gram	Neisser	Growth on serum	Growth on agar	Growth on gelatine
-	Throat in diphtheria	Diphtheria bacillus	+	+	...	...	...
G13	In pus from T.B. abscess of hip	On serum banded and mostly of D1 type; on agar show single median septum	+	-	Greyish-white growth of small, circular, discrete, colonies	Thick growth of raised, circular, discrete, greyish-white colonies	Good growth in 5 days of raised, circular, white colonies
S19	Ear in scarlet fever	Short banded bacillus of E1 type; very well-marked polar granules	+	+	Minute, circular, raised, discrete, white colonies, like K.L., but smaller	Greyish, film-like growth of minute, circular, flat colonies	No growth
G5	Urine in T.B. cystitis	Short, slender, banded bacillus, mostly of E1 type	+	-	Cream-coloured, dry, minute, circular colonies; very adherent to medium	Minute, raised, white colonies with ground-glass surface	Very slow growth of minute colonies
C16	Throat in diphtheria	Mostly solid staining or of D2 type	+	-	Pure yellow, thick, confluent growth	Yellow colonies with crenated edges	Pale yellow, raised colonies in 30 hours
G8	Urethral pus (3 strains)	Irregular, curved, banded bacilli of A1 and C1 types; many clubbed	+	+	Minute, white colonies like K.L., but smaller	Very minute, greyish-white, circular colonies	No growth
G6	Urine in T.B. cystitis	Short, oval bacillus with single, unstained septum; some banded and clubbed; arrangement marked	+	+	Very like diphtheria bacillus	Good white growth, like diphtheria	No growth
S13	Ear in scarlet fever (4 strains)	Long, narrow, clubbed and curved bacilli, banded and of A1 type	+		Small, raised, white, circular colonies, like K.L.	Film-like growth of very minute, greyish, circular colonies	No growth
G12	Vagina in vaginitis	Small, plump, oval bacilli of D2 type, with single median septum; later most are banded and of D1 type	+	+ Very scanty granules	Small white colonies, like K.L.	White colonies, like K.L.	Like K.L. in 36 hours
D1	Throat in diphtheria (2 strains)	Very like <i>Bacillus Hofmanni</i> of D2 type	+		Pure yellow, raised, circular colonies, like K.L., except in colour	Like K.L., but pure yellow	Discrete small colonies, yellow

[illegible]

	Source	Morphology	Gram	Neisser	Growth on serum	Growth on agar	Growth on gelatine
X	Not known	Short, slender bacilli, with single median septum; few banded	+	+	Growth like K.L.	Thick, greyish-white growth of circular colonies	...
S79	Ear in scarlet fever	Short, thick bacilli of D2 type; few banded forms	+	-	Growth like K.L.	Raised, white, circular colonies	...
S5	Ear in scarlatinal otitis media (3 strains)	Long, slender, banded and clubbed of A1 and C1 types	+	+	Thick, confluent, moist, pink growth	Large, circular, raised, pink colonies	No growth
G16	Vagina in vulvitis	Medium-sized banded and clubbed bacilli, mostly of D1 type	+	+	Very minute greyish-white circular colonies	No growth in 24 hours; later, very minute greyish, circular colonies, with filmy edges	No growth
G4	Eye in hordeolum	Medium length, fairly thick, most of D2 type, some banded	+	+	Raised, circular, discrete colonies like K.L., but smaller	No growth in 24 hours; later, very minute greyish-white, circular colonies	Very minute colonies after some days
B	Skin in leprosy	Very irregular, bacilli mostly of A type; clubbing very marked, also polar granules	+	+	Raised, white, circular colonies, like K.L.	Very minute, circular, greyish-white colonies	...
S1	Ear in scarlet fever (3 strains)	Nearly all of D2 type; few banded forms (<i>Bacillus Hoffmanni</i>)	-	-	Raised, circular, white colonies like K.L.	Raised, white, circular colonies 1 mm.	...
E	Normal ears and ears in scarlet fever (10 strains)	Medium length, slender bacilli, solid staining, with well-marked polar granules, some with one or several septa (<i>Bacillus ceruminis</i>)	+	+	Growth very like K.L.	Minute creamy-white colonies	No growth
S38	Ear in scarlet fever	Long, slender, banded bacilli, marked terminal granules	-	+	Like K.L.	Thick, white, waxy-looking growth	...
S42	Nose in scarlet fever	Short, thick bacilli, some banded, others with single septum	+	-	Very faint, white growth	No growth in 24 hours; later, very minute circular colonies	...

	Source	Morphology	Gram	Neisser	Growth on serum	Growth on agar	Growth on gelatine
S58	Ear in scarlet fever	In 48 hours on serum; medium length, slender, curved bacilli, with very numerous granules	+	+	Very minute white colonies	No growth in 24 hours; later, very minute colonies	..
S95	Ear in scarlet fever (2 strains)	Slender bacilli, mostly solid staining; few banded, well-marked polar granules	+	+	Creamy-white confluent growth	Large, circular, white colonies	...
G20	Unknown	Mostly clubbed bacilli of A and A1 types; very prominent polar granules	+	+	Creamy-white circular colonies	Filmy growth of minute, circular colonies	...

VIRULENCE TESTS.

The most useful of all the ordinary laboratory methods for distinguishing true diphtheria bacilli from bacilli resembling them is the virulence test. This, however, does not serve to distinguish between avirulent diphtheria bacilli and avirulent diphtheroids. Several authors [4, 5, 6] have described diphtheroid organisms which are virulent and kill laboratory animals, but the animals are not protected by diphtheria antitoxins.

The cultural characteristics of the diphtheria bacillus on serum and other media are much too variable to be of great use in the differentiation of the true diphtheria bacillus from diphtheroids. For each medium, however, there is a form of colony which the diphtheria bacillus most commonly presents.

On examination of the table it will be seen that several of the diphtheroids investigated presented growths very much resembling the typical growths of the diphtheria bacillus. It should be added that the cultures were grown on serum, agar, and broth for twenty-four hours at 37° C., unless otherwise stated. The fermentation reactions given in the table were observed in Hiss's serum water medium [96], kept for ten days to a fortnight at 37° C. The production of acid was in all cases, however, evident within the first forty-eight hours.

In describing the morphology of the diphtheroid organisms use has

Growth in broth	Growth on potato	Dextrose	Lactulose	Saccharose	Lactose	Maltose	Mannite	Dulcife	Glycerine	Dextrine	Galactose	Milk	Virulence
No growth	...	...	...	...	...	...	...	...	...	...	...	...	...
Slight turbid- ity, with de- posit	...	...	...	...	...	...	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...	...	...	...	...	...	...

been made of the classification of Wesbrook, Wilson, and McDaniel [97], for it is more convenient and more exact than the usual morphological descriptions.

It is obvious that the results obtained are all of a negative character, and go to show that there is no one reliable method at present in use which will in all cases without exception distinguish the true diphtheria bacillus from diphtheria-like bacilli other than *Bacillus Hofmanni*. Further, the present bacteriological methods are not sufficient to give a basis of classification of the diphtheroid organisms among themselves. The diphtheroid organisms have been shown to be almost as ubiquitous as the streptococci, and to present as great difficulties in their differentiation and classification.

CONCLUSIONS.

(1) Forty-nine strains of diphtheroids have been obtained from various parts of the human body, and examined by the usual bacteriological methods.

(2) Thirty-three of these strains were isolated from the external auditory meatus, eight from normal persons and twenty-five from persons suffering from scarlatina.

(3) It was found that there was no definite type of diphtheroid organisms peculiar to the ears of scarlatina patients. There is, however,

apparently a definite type of diphtheroid occurring in the ears of normal persons.

(4) The fermentation reactions were not found to be a very satisfactory method for the separation of the different strains of diphtheroids included in this research from one another, and from the true *Bacillus diphtheriæ*. In particular the fermentation of glucose is of little value.

(5) Serological tests were without exception unsatisfactory.

(6) For the differentiation of the diphtheroid organisms from one another, and from the true *Bacillus diphtheriæ*, all the cultural characteristics must be considered.

In conclusion, I wish to thank Dr. Eyre, Dr. Thompson, Dr. Arkwright, and Dr. Bayon for material, and Dr. Henderson Smith for the assistance he has given me during the course of the investigation.

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XLIII. SECOND REPORT ON INVESTIGATIONS INTO PLAGUE VACCINES.

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With 1 Diagram.

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IN a former report (this *Journal*, Vol. x. p. 536) some of the contents of plague bacilli which had been killed by chloroform were extracted by two methods: (1) simple digestion of the organisms with saline, (2) treatment of the moist bacteria with enough anhydrous sulphate of soda to combine with all the water present, freezing and

thawing the mixture by changing the temperature from 18° C. to 37° C.¹, filtering off the bacterial deposit at 37° C. and subsequent extraction of the bacteria with water.

The two solutions so obtained possess much the same chemical properties. Both contain one or more kinds of nucleoprotein, accounting for about two-thirds of the total nitrogen in the extract. As regards their physiological properties, however, there is a striking difference. The first extract, in the previous report called solution A, has only a small degree of toxicity for rats and, by the method employed (this *Journal*, Vol. x. p. 545), its immunising value was shown to be negligible. On the other hand, the second extract, obtained after treatment with sodium sulphate and called solution B, was fatal to rats, 0·05 to 0·1 mg. killing them in 18 hours: doses of 0·001 to 0·01 mg. afforded substantial protection, the latter amount reducing the mortality after the inoculation of a standard dose of plague culture from 80 % to 10 %. The toxic and immunising properties always accompanied the nucleoprotein throughout the various chemical manipulations to which it was subjected. The residue left after treatment with sodium sulphate and extracting with water was shown to be devoid of acute toxicity and immunising properties.

I. THE ACTION OF CHLOROFORM.

The cause of the discrepancy in physiological activities of these two chemically similar extracts was not clear at the time of my previous report. I was inclined to believe that the toxic and immunising properties resided in some substance other than the nucleoprotein which for some unexplained reason was extracted together with it only after treatment with the sodium sulphate. Since then I have been able to clear up the mystery and at the same time to explain the different conclusions arrived at by Pick and myself with regard to the effect of chloroform upon the antigen of the plague bacillus.

It is stated by Pick (Kraus and Levaditi's *Handbuch*, Bd. i. p. 353) that chloroform destroys the antigen of the plague bacillus and in the first of these reports I explained that I had not been able to confirm this statement but had found that a toxic solution possessed of good immunising power could be prepared from organisms that had been

¹ Na_2SO_4 changes to $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ between these temperatures. At 37° C. the mixture exists as an emulsion of bacteria in a saturated solution of Na_2SO_4 , at 18° C. as a hard rocky mass of Glauber's salt and dry bacteria.

killed by chloroform. The explanation of these contradictory statements is afforded by the following experiments which show (1) that chloroform combines with some substance in the extract giving rise to a relatively atoxic modification; (2) that this compound is reversible; and (3) that a ready method of reversing the combination and of reinstating the toxicity is afforded by the use of anhydrous sodium sulphate.

EXP. 1. Sixty grammes of "sulphated" bacilli¹ were taken and divided into two portions of 30 grammes each. The sulphate was removed from both portions by adding just sufficient water to dissolve it at 37° C. and filtering. The pasty masses of bacilli left on the filter were treated as follows:

part 1 was extracted for 3 hours in chloroform water,

part 2 was extracted for 3 hours in distilled water: both at 37° C.

Both portions were then filtered and the nucleoprotein in the filtrate determined as usual. Both extracts contained the same quantity of nucleoprotein. The lethal dose of the nucleoprotein of the first solution was 2 mg., of the second 0.1 mg. The chloroform water extract although containing the same amount of nucleoprotein only possessed $\frac{1}{20}$ th the toxicity of the water extract. This might be explained on the assumption that the nucleoprotein was not the active agent and the following experiment supports this interpretation.

EXP. 2. The same experiment was repeated with the modification of adding chloroform to the water extract after the completion of the extraction. The results are given below and show that the toxicity of the solution was unaltered. The degrading effect of chloroform on toxicity is thus greatest at the moment of extraction.

Details of experiments 1 and 2 are given in Table I.

In my previous report it was shown that the toxic and immunising properties of the extract prepared by the "sulphate process" followed so closely the content of nucleoprotein that it was difficult to believe that they belonged to some merely adherent substance and not to the nucleoprotein itself.

The following experiments, which show that the action of chloroform

¹ Wherever possible, in the course of this work, the various extracts have been made from the same batch of "sulphated" bacilli, a large stock of which, several kilogrammes, was prepared, thoroughly mixed and kept in the ice box. The method of preparation of this powder of "sulphated" bacilli is given in the last report in full. Briefly it is the washed surface growth from agar set in Roux flasks, mixed with anhydrous sulphate of soda and ground to a fine powder. Once prepared it apparently preserves its properties indefinitely and is a most convenient means of ensuring a constant supply of plague bacilli.

on the nucleoprotein is reversible, completely explain the apparent inconsistency of the results of experiments 1 and 2 with this view.

EXP. 3. A quantity of an emulsion of organisms was prepared and a few cubic centimetres of chloroform added to the flask so that some remained free and undissolved at the bottom of the flask. The emulsion

TABLE I. *Experiment 1.*

<i>Toxicity of the extract made in chloroform water.</i>			<i>Toxicity of the water extract.</i>		
Dose, mgs.	Rat	Result	Dose, mgs.	Rat	Result
1·7	(1	* + 1 day	1·7	(1	+ 1 day
	(2	0		(2	+ 1 day
0·8	(3	0	0·8	(3	+ 1 day
	(4	0		(4	+ 1 day
0·4	(5	0	0·4	(5	+ 1 day
	(6	0		(6	+ 1 day
0·2	(7	0	0·2	(7	+ 1 day
	(8	0		(8	+ 1 day

* The symbol + is used throughout this report to indicate the death of the animal, 0 its survival.

Experiment 2. Toxicity of the water extract to which chloroform was added.

Dose, mgs.	Rat	Result
1·7	(1	+ 1 day
	(2	+ 1 day
0·8	(3	+ 1 day
	(4	+ 1 day
0·4	(5	+ 1 day
	(6	+ 2 days
0·2	(7	+ 2 days
	(8	—

TABLE II.

Experiment 3. Determination of toxicity.

Dose, mgs.	(1) Emulsion in chloroform water		(2) Same emulsion after pumping out the chloroform	
	Rat	Result	Rat	Result
1·0	(1	0	1	+ 1 day
	(2	0	2	+ 1 day
0·8	(3	0	3	+ 1 day
	(4	0	4	+ 1 day
0·6	(5	0	5	+ 1 day
	(6	0	6	+ 1 day
0·4	(7	0	7	+ 1 day
	(8	0	8	+ 1 day
0·2	(9	0	9	+ 1 day
	(10	0	10	+ 1 day

was divided into two parts. Both parts were incubated at 37° C. for 6 hours. Part 1 was set aside, while part 2 was exhausted by the Fleuss pump for some hours until no smell of chloroform was noticeable. Both parts were left till the next day when they were filtered and the nucleoprotein content and toxicity determined (see Table II).

The lethal dose of the pumped out portion was 0.2 mg., possibly less, while that from which the chloroform had not been so removed was greater than 1 mg. The amount of nucleoprotein was the same in each.

EXP. 4. A quantity of organisms killed by chloroform were mixed with sulphate of soda in the usual way and the sulphate removed by dissolving with water at 37° C. As we have seen, this process yields organisms free from chloroform and in such a condition that the nucleoprotein with its toxic and antigenic properties can be extracted with water from the bacilli. In this case chloroform was added to the water with which the paste of organisms was extracted. The chloroform water extract was comparatively toxic, although it contained the usual amount of nucleoprotein. On pumping out the chloroform, however, the solution was again rendered toxic to the usual degree (M.L.D. = 0.1 mg.). After this extraction the organisms were again mixed with sulphate which in its turn was removed. The paste of organisms left after this second sulphating was extracted in water and a small yield of nucleoprotein again obtained, of which the lethal dose was as usual about 0.1 mg. (see Table III).

These experiments show that the atoxic nucleoprotein extracted by means of chloroform water can be rendered toxic by the simple process of removing the chloroform in a vacuum. There is, therefore, no reason to suppose, as was done in my previous report, that the nucleoprotein extracted by water from the organisms killed by chloroform (nucleoprotein A in the last report) is not the same substance as that extracted from similar material after sulphating (nucleoprotein B of last report). The nucleoprotein A is in combination with chloroform, and for this reason has lost its toxic properties which, however, can be reinstated by removing the associated chloroform with the air pump. It is further clear that one of the effects of the sulphate of soda used in the process is to remove this chloroform from its loose combination with the nucleoprotein.

This removal of chloroform by the sulphate of soda appears to be due to evaporation of the chloroform following on the withdrawal of all the water by the anhydrous salt to form $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$.

TABLE III. *Toxicity determinations.*

Dose mgs.	Chloroform water extract		Chloroform water extract after pumping out the chloroform	
	Rat	Result	Rat	Result
2.5	{ 1	0	1	+ 1 day
	{ 2	0	2	+ 1 day
1.25	{ 3	0	3	+ 1 day
	{ 4	0	4	+ 1 day
0.5	{ 5	0	5	+ 1 day
	{ 6	0	6	+ 1 day
0.25	{ 7	0	7	+ 1 day
	{ 8	0	8	+ 1 day

Dose, mgs.	Second water extract	
	Rat	Result
1.1	{ 1	+ 1 day
	{ 2	+ 1 day
	{ 3	+ 1 day
0.56	{ 4	+ 1 day
	{ 5	+ 1 day
0.28	{ 6	+ 2 days
	{ 7	+ 3 days
0.05	{ 8	+ 3 days

Why the effect of chloroform upon the toxicity and immunising power of the nucleoproteins should be so different, according to whether it is brought into play at the moment of extraction or subsequently (as in Experiment 1), still remains unexplained. It may well be that the composition of these nucleoproteins at the moment of soaking out from the recently killed bacillus is somewhat different to that which obtains afterwards. Nucleoproteins are bodies with great possibilities of combination with other proteins and there is no evidence that they exist as such inside the living cell.

An interesting point with regard to the reversibility of the chloroform-nucleoprotein-toxin combination emerges from the study

TABLE IV.

Delayed mortality, using chloroformed nucleoprotein.

Dose, mgs.	Rat	Result
1.6	{ 1	+ 1 day
	{ 2	0
0.83	{ 3	+ 8 days
	{ 4	+ 8 days
0.4	{ 5	+ 5 days
	{ 6	+ 4 days
0.2	{ 7	+ 27 days
	{ 8	+ 10 days

of the effect of this substance when inoculated into rats. The lethal dose (in this work the death of the animal in two days is regarded as determining the L.D.) is large (1 to 2 mg.) but there is observed a delayed mortality amongst the animals corresponding to a gradual dissociation of the compound after inoculation. Thus in one experiment the results in Table IV were obtained.

It will be a matter of interest to follow up this delayed toxic action in the body since its bearing on the problem of vaccination is apparent.

The effect of toluol on the toxicity and immunising properties of the nucleoprotein.

Toluol exerts but slight deleterious influence upon the activity of solutions of the nucleoproteins of the plague bacillus and was found to be the best preservative to use during their preparation. I tried therefore whether this substance could be advantageously employed instead of chloroform vapour to kill the bacilli.

Preliminary experiments were made to determine the time necessary to kill the organisms. For this purpose a good surface growth on agar in a Roux flask was used. The flask was inverted and a few cubic centimetres of toluol introduced. At the end of each quarter of an hour a whole loopful of the growth was withdrawn from the flask and inoculated on the surface of agar. In this way the following results (Table V) were obtained :

TABLE V.

Showing the presence or absence of growth in the tubes inoculated from the flask after exposure to toluol vapour for the times indicated.

Quarter hour intervals	Growth after		
	24 hours	48 hours	72 hours
1	+	+	+
2	+	+	+
3	+	+	+
4	-	+	+
5	-	+	+
6	-	+	+
7	-	-	-
8	-	-	-
9	-	-	-
10	-	-	-
11	-	-	-

These preliminary experiments showed that toluol can be used as a germicide in the same way as chloroform (viz. inverting the Roux flask and introducing a few c.c. of the liquid into the flask, thus exposing the bacilli to the action of the vapour only), about one and a half hours being required to completely sterilize a Roux flask. Seventy-three Roux flasks were treated in this way. Of the emulsion obtained, one half was left in contact with toluol for two days, the other half was centrifugalised and washed as usual. After washing, the latter was sulphated in the ordinary way, and a water extract corresponding to solution B obtained; of this solution 0.2 mg. nucleoprotein was a lethal dose. The portion of the emulsion left in contact with the toluol was filtered, and of the fluid obtained the lethal dose was about 1.0 mg.

Toluol apparently behaves similarly to chloroform and forms a loose combination with the nucleoprotein, the combination being less toxic. By treatment with anhydrous sulphate of soda a more toxic product is obtained (Table VI).

TABLE VI.

<i>Toxicity of toluol water extract.</i>			<i>Toxicity of water extract from toluol-killed and sulphated organisms.</i>		
Dose, mgs.	Rat	Result	Dose, mgs.	Rat	Result
1.5	(1	+ 3 days	4.0	(1	+ 1 day
	(2	0		(2	+ 1 day
0.6	(3	+ 3 days	2.0	(3	+ 1 day
	(4	0		(4	+ 1 day
0.3	(5	+ 3 days	1.0	(5	+ 1 day
	(6	0		(6	+ 1 day
0.15	(7	0	0.4	(7	+ 1 day
	(8	0		(8	+ 1 day
			0.2	(9	+ 1 day
				(10	+ 2 days

II. ON THE PRESENCE OF A HYDROLYTIC ENZYME IN THE PLAGUE BACILLUS.

A quantity of plague bacilli were grown on agar in Roux flasks. They were killed by chloroform vapour and an emulsion made in salt solution: 8% of the nitrogen of the emulsion was soluble in saturated tannic acid¹. The emulsion was incubated under toluol at 37° C.

¹ This method of determining the progress of hydrolysis is due to Hedin (Hedin and Rowland, "Ueber ein Proteolitiches Enzym in der Milz," *Zeitschr. f. phys. Chem.* Bd. xxxii. p. 342). The precipitation of the undigested proteids is effected by the addition of an equal volume of a solution of tannic acid 7% and acetic acid 5%.

After 24 hours 28 % of the total nitrogen was in a form which was not precipitated by tannic acid. In 6 days 38 % was reduced to this form, while in 10 days 50 % was so changed.

The experiment was repeated with the addition of 11 % sulphate of soda to the emulsion. In this case 38 % of the nitrogen was converted in 24 hours and 48 % after 10 days.

The solution contained 780 milligrammes of protein in 100 c.c. The quantities found after the lapse of the times indicated are given in Table VII.

TABLE VII.

Time	Protein found, mgs. per 10 c.c.
As prepared	78
18 hours	68
45 hours	49
65 hours	49
22 days	38

The diminution of the nitrogen precipitable by tannic acid and of the total proteins coagulable by heat runs parallel, from which it is concluded that the bulk of the protein hydrolysed by the enzyme is derived from the nucleoprotein. The great falling off in rate as the hydrolysis proceeds at the stage when less than 50 % of the total protein has been attacked, suggests either that the enzyme is readily destroyed, or that only a proportion of the protein is capable of hydrolysis by this means. The effect of the hydrolysis by this enzyme upon the toxicity and immunising power of the nucleoprotein extract will be dealt with directly (p. 35 below).

There exists, therefore, in the plague bacillus an enzyme capable of hydrolysing the protein. For the sake of comparison with a phenomenon which is better known, an experiment was made with some brewer's yeast under the same conditions. Here after 5 days 73 % of the nitrogen was converted, while in the presence of sulphate of soda (10 %) 59 % was changed.

The activity of this enzyme is therefore comparable with that of a proteolytic ferment of recognised power.

The question now arises, is this enzyme extracted by the methods employed in preparing an actively immunising solution by the sulphate process? A quantity of solution of nucleoproteins was prepared by dehydration with Na_2SO_4 and extraction in saline and examined in the same way. The percentage of nitrogen hydrolysed to the extent of being soluble in tannic acid mixture is shown in Table VIII.

TABLE VIII.

The solution contained 7.8 mgs. of nucleoprotein per c.c.

Time	Percent. nitrogen not precipitable by tannic acid mixture after various intervals at 37° C.
As prepared	5.7
18 hours	29
45 hours	41.7
65 hours	44
22 days	56

Concurrently with this hydrolysis there was a loss in protein as estimated by boiling after acidulation with acetic acid.

III. THE EFFECT OF HEATING SOLUTIONS OF THE NUCLEOPROTEIN ON THEIR IMMUNISING POWER, TOXICITY AND THE ACTIVITY OF THE HYDROLYTIC ENZYME CONTAINED THEREIN.

As shown by Famulener and Madsen (*Biochem. Zeitschr.* Bd. XI. p. 186, 1908), and by Martin and Chick (*Journ. Physiol.* Vol. XL. p. 404, 1910), the effect of hot water upon certain antigens (vibrio-lysin, tetano-lysin) and upon proteins does not occur instantaneously when a particular temperature is reached. It is a time process in which heat merely plays the subsidiary part of an accelerator. The action has an extraordinary high temperature coefficient and the rate of the reaction is increased in some cases as much as twice per degree centigrade rise in temperature.

Famulener and Madsen studied the effect of hot water upon vibrio-lysin and tetano-lysin and were able to obtain data for the calculations of the antigen destroyed as time elapsed by test tube experiments.

In the case of plague antigen, however, *in vitro* experiments are not feasible and the number of animal experiments required to determine each point would be very costly. I have therefore been obliged to content myself with observations indicating the fall in antigenic power suffered by the minimal optimal immunising dose by heating for half an hour to various temperatures. The diminution in immunity conferred was indicated by the percentage of animals which survived a subsequent inoculation of 0.1 c.c. of the standard plague culture after they had received the same quantity of antigen solution heated at each temperature. What the relation between the percentage protected and

proportion of antigen remaining after heating may be, is at present being determined.

As stated in my previous report (this *Journal*, Vol. x. p. 559), the protection afforded by a single dose of one-hundredth of a milligramme of the nucleoprotein and associated antigen is to protect from 80–90 % of the rats inoculated against a subsequent dose of virulent plague such as will kill 80 % of the control animals. The following table gives the protection afforded by a similar dose of nucleoprotein which had been heated to the temperatures named for half an hour.

TABLE IX.

Temp. to which antigen solution was heated (°C.)	No. of rats in series	Mortality	% Mortality
Unheated	19	2	11
55	20	6	30
60	36	18	50
64	39	21	54
65	38	30	79
Controls inoculated with test culture only	100	—	78

In the last series a precipitation took place which was filtered off, the clear fluid only being inoculated.

This experiment shows that the destruction of antigen is appreciable below 55° C. and that the rate increases slowly until 64° C. is reached. At 65°, at which temperature the nucleoprotein was precipitated¹, the whole of the antigen disappeared from the solution, for the mortality was the same as in the controls.

1. *The effect of heating upon the toxicity of solutions of nucleoprotein.*

A quantity of toxic nucleoprotein solution was prepared and heated in the water bath to various temperatures for half an hour. Table X below shows the experimental results of inoculating these heated solutions into rats.

The minimal fatal dose of this sample of the nucleoprotein was evidently just above 0.1 mg., for this quantity of the unheated material killed the two rats in two and three days respectively. Heating to 53° C. and 60° C. for half an hour reduced the toxicity to $\frac{1}{4}$ th (0.4 mg. = M.L.D.) and $\frac{1}{3}$ th (0.8 to 1 mg. = M.L.D.) respectively. The observations at 56° C. only give an upper limit.

¹ The precipitation of the nucleoprotein by heat does not always occur at this temperature. The exact point is dependent upon the reaction and concentration of the solution.

From the series at 53° C. and at 60° C. some idea of the acceleration of the destruction per degree rise of temperature can be obtained. Taking 0.4 mg. as the minimal lethal dose after heating to 53° C. for half an hour and 0.9 mg. as the mean value after heating to 60° C. for the same period, the relative rates of destruction are as 9 to 4 for a rise of 7° C. This gives a temperature coefficient of 3.2 per 10° C.

TABLE X.

The solutions were of the same reaction and nucleoprotein concentration and were heated to the temperatures for half an hour.

Dose, mgs.	Rat	Kept in ice box	53° C.	56° C.	60° C.
4.0	(1	...	...	+ 1 day	...
	(2	...	...	...	+ 3 days
2.0	(3	...	+ 1 day	+ 1 day	...
	(4	...	+ 1 day	+ 1 day	...
1.0	(5	...	+ 1 day	+ 1 day	+ 3 days
	(6	...	+ 1 day	+ 3 days	0
0.8	(7	...	+ 1 day	...	+ 3 days
	(8	...	0	...	0
0.6	(9	+ 1 day	+ 1 day	+ 1 day	0
	(10	+ 1 day	+ 1 day	+ 1 day	0
0.4	(11	+ 1 day	+ 1 day	...	0
	(12	+ 1 day	0	...	0
0.2	(13	Very ill	...	...	...
	(14	Very ill	...	...	...
0.1	(15	+ 2 days	...	...	...
	(16	+ 3 days	...	...	...

2. *The effect of heating solutions of the nucleoprotein upon their hydrolytic activity.*

Like other enzymes, the hydrolytic ferment in the plague bacillus is destroyed by heating its solution. It is impossible to properly determine the influence of temperature upon the rate of the destruction of the enzyme by hot water, because, so far, I have not succeeded in separating the enzyme from the "substrate." Some qualitative notion of the influence of hot water was, however, obtained by heating solutions of the nucleoprotein containing the enzyme for half an hour at various temperatures, and estimating the amount of non-precipitable nitrogen after two days' subsequent incubation at 36° C. (Table XI).

As this method does not give an estimate of the work done by the enzyme after heating irrespective of the increased work done by the enzyme during the heating, inasmuch as it is not possible to instantaneously bring the solution to the temperature required, the experiment

was repeated with a series of controls in which the changed nitrogen was estimated immediately after the heating. The results are given in Table XII.

TABLE XI.

Temp. to which the solution was heated	Percent. nitrogen not precipitated by tannic acid mixture
Control (not heated)	36·8
51—52° C.	33·8
53—54	30·4
55—56	24·5
59—60	16·6
63—64	13·0

TABLE XII.

Temp. to which the solution was heated for 30 mins.	Amount of nitrogen in non-precipitable form	
	Determined at once	After two days
Control	10·9 %	49·5 %
39° C.	12·1	—
44	—	47·9
50	14·7	—
55	15·0	40·0
58	15·1	—
63·5	13·1	17·4

These determinations indicate that on heating for half an hour the temperature at which the first damage to the enzyme can be recognised is about 50° C.

3. *Comparison of the effect of heating the bacilli on the toxicity and immunising power of the extract subsequently obtained from them, with the effect of heat applied to the extract itself.*

A. *With regard to toxicity.*

For this experiment 100 Roux flasks of plague bacilli were prepared as usual, but instead of being chloroformed they were heated to 60° C. in the steamer. The steamer was first raised to this temperature, then opened, the flasks introduced and when it had again reached 60° C. the time was noted and heating continued for half an hour. The surface growth was washed off as usual in salt solution and the emulsion centrifugalised. The first washings, *i.e.* the salt solution that had been in contact with the bacilli for some two or three hours, in contrast to what occurred in the case of bacilli killed by chloroform, contained no

nucleoprotein in solution. The paste of organisms was "sulphated" in the usual way, the mass left to cool and placed at 37° C. for the night. Next morning enough water at 33° C. to dissolve the sulphate was added and the whole placed on a hardened filter. The residue of organisms left on the filter was extracted with water. The suspension would not filter and rapidly became a slimy mass resembling sputum. It was accordingly centrifuged and an opalescent fluid obtained. The amount of nucleoprotein obtained was equal to that usually yielded by similar treatment of chloroform killed, *i.e.* unheated, bacilli. The nucleoprotein obtained by this process was of about the same toxicity as that obtained after killing the bacilli with chloroform, 0.2 mg. being an acute lethal dose.

In view of the effects of heating the toxin already given, the fact that this solution was highly toxic indicates that there is a considerable difference in the effect produced by heating it in solution in water and heating it in the condition in which it occurs within the body of the bacillus.

TABLE XIII.

Lethal dose of extracted nucleoprotein heated to 55° C. for half an hour.

Dose, mgs.	Rat	Result
2.0	{ 1	+ 1 day
	{ 2	+ 1 day
1.0	{ 3	+ 1 day
	{ 4	+ 2 days
0.6	{ 5	+ 1 day
	{ 6	+ 1 day
0.4	{ 7	0
	{ 8	0

Lethal dose of an emulsion heated to 55° C. for half an hour.

Dose, mgs.	Rat	Result
2.0	{ 1	+ 1 day
	{ 2	+ 2 days
1.0	{ 3	+ 1 day
	{ 4	+ 3 days
0.5	{ 5	0
	{ 6	0

The difference between the effect of heat upon the toxicity of the extracted nucleoprotein and of the bacillus respectively was further studied by comparing the toxicity of the extracted nucleoprotein heated to 55° C. with that of organisms heated to the same temperature.

In order to institute a comparison between the toxicity of the whole bacilli and that of the extracted nucleoprotein it is necessary to know what proportion of the bacillus the latter comprises. From previous observations (this *Journal*, Vol. x. p. 536) it was found that the nucleoprotein constitutes about one-tenth by weight of the whole bacillus. The toxicity of the extracted nucleoprotein solution after heating to 55° C. for half an hour was found by experiment to be between 0.5 and

1 mg. On the assumption that the effect of heat upon toxicity when the bacillus is heated as a whole is the same as when the toxic nucleoprotein is heated after extraction, the lethal dose of whole bacilli heated to 55° C. should be 6·0 mg. whereas it was about 1 mg. The protocols of this experiment are given in Table XIII.

The contrast is still more marked if a comparison be made between the toxicity of the nucleoprotein solution made from unheated organisms and a similar solution made from organisms that have been heated to 60° C. for half an hour, a temperature which we have seen reduces the toxicity of the nucleoprotein solution to one-ninth. At a temperature at which the toxicity of a solution in water is greatly reduced, only a small diminution occurs when the toxic nucleoprotein is in the cell, as the following experiment shows.

TABLE XIV.

<i>Toxicity of nucleoprotein solution heated to 60° C. for half an hour.</i>			<i>Toxicity of nucleoprotein solution prepared from organisms that had been heated to 60° C. for half an hour.</i>		
Dose, mgs.	Rat	Result	Dose, mgs.	Rat	Result
4·0	{ 1	+ 5 days	0·8	{ 1	+ 1 day
	{ 2	0		{ 2	+ 1 day
1·0	{ 3	+ 3 days	0·4	{ 3	+ 1 day
	{ 4	0		{ 4	+ 2 days
0·8	{ 5	+ 3 days	0·2	{ 5	+ 2 days
	{ 6	0		{ 6	+ 2 days
0·6	{ 7	0	0·1	{ 7	0
	{ 8	0		{ 8	0

The lethal dose of the toxin prepared from unheated organisms is about 0·1 mg.

I have at present no explanation to offer to account for the difference in the rate of destruction of the toxin according to whether it is heated whilst within the bacillus or after extraction. The very large effect of small changes in the concentration of hydrogen ions and variations in the concentration of salts upon the rate with which proteins are attacked by hot water recently described by Chick and Martin, *Journal of Physiol.* Vol. XL. p. 404, 1910, suggests a possible explanation. This subject is at present under investigation, because of the practical importance of any facts which may lead to a differential method of obtaining the antigen and toxin which are apparently bound up in the nucleoprotein.

B. *With regard to immunising power.*

A comparison of the effect of heat upon the immunising properties of the nucleoprotein (1) whilst within the bacillus and (2) subsequent to extraction, indicates that under the former circumstances a similar diminution in rate of destruction occurs to that found in the case of toxicity.

An emulsion of organisms was prepared and killed by heating to 55° C. for half an hour. Of 34 rats that had received such a volume of this emulsion as contained 0.01 mg. of dry bacterial substance, 31 survived the subsequent test inoculation with plague. The amount of nucleoprotein contained in the quantity of organisms inoculated was (as was shown in the previous report, this *Journal*, Vol. x. p. 536) about one-tenth by weight, or 0.001 mg.

A second series of 20 rats were inoculated with ten times this quantity of extracted nucleoprotein that had been heated to 55° C. for half an hour precisely as was the emulsion used in the last experiment. Of these rats only 14 survived the subsequent test inoculation. There is here an ~~increase~~ of 21 per cent. of survivals in the case where the antigen had been heated inside the bacillus; and this increase in face of the fact that the dose of antigen was one-tenth as great¹.

IV. THE EFFECT OF HYDROLYSIS ON THE TOXICITY AND IMMUNISING PROPERTIES OF THE NUCLEOPROTEIN SOLUTION.

1. *Effect on toxicity.*

As mentioned on p. 27 above the watery or dilute saline extract of plague bacilli killed by chloroform vapour contains a hydrolytic enzyme. It had been noticed in the course of this investigation that the toxicity of an extract diminished day by day when kept at laboratory temperatures and in view of the fact recorded by Ruffer and Wilmore² that the endotoxin obtained from the dysentery bacillus is destroyed by digestion with pepsin, it was considered likely that this might be accounted for by the action of the enzyme. The following experiment shows that the

¹ That it was really one-tenth is a deduction based on the nucleoprotein content of the two vaccines. Until the relation between antigen injected and survivals has been worked out so as to afford a biological test for the amount of antigen, this is the only quantitative method of indicating the amount of antigen we possess.

² *British Medical Journal*, 1908, Vol. II. p. 1176.

progressive hydrolysis of the nucleoprotein on incubating the solution at 37° C., described on p. 28 above, is accompanied by a loss of toxicity. This is shown in Tables XV and XVI below. The extract was slightly alkaline to litmus paper.

TABLE XV.

The original solution contained 7·8 mg. of nucleoprotein per cubic centimetre.

Time incubated at 37° C.	Lethal dose *
Fresh	0·05 c.c.
18 hours	about 0·4
45 hours	more than 1·0
65 hours	„ 7·0

* Portions removed from time to time to test toxicity were diluted 1 in 7·8.

TABLE XVI.

Protocols for determination of toxicity experiment in Table XV above.

Fresh solution			Solution incubated for 18 hrs.			Solution incubated for 45 hrs.		
Dose, c.c.	Rat	Result	Dose, c.c.	Rat	Result	Dose, c.c.	Rat	Result
0·3	{1	+ 1 day	0·4	{1	+ 1 day	1·0	{1	0
	{2	+ 2 days		{2	0		{2	0½
0·2	{3	+ 1 day	0·3	{3	+ 2 days	0·8	{3	0
	{4	+ 2 days		{4	0		{4	0
0·1	{5	+ 1 day	0·2	{5	0	0·6	{5	+ 1 day
	{6	0		{6	0		{6	0
0·05	{7	+ 1 day	0·1	{7	0	0·4	{7	0
	{8	+ 2 days		{8	0		{8	0

Other determinations of the same point are here given (Tables XVII–XX). In these cases the solutions were kept at the temperature of the laboratory.

TABLE XVII.

The original solution contained 3·5 mg. of nucleoprotein per cubic centimetre.

Age	Lethal dose *
1 day	0·1 c.c.
24 days	1·0
54 days	more than 2·0
72 days	about 7·0

* Portions removed from time to time to test toxicity were diluted 1 in 3·5.

TABLE XVIII.

Protocols of experiments in determination of toxicity in Table XII above.

Fresh solution.			Solution incubated for 24 days.			Solution incubated for 54 days.		
Dose, c.c.	Rat	Result	Dose, c.c.	Rat	Result	Dose, c.c.	Rat	Result
0·6	{ 1	+ 1 day	4·0	{ 1	+ 1 day	7·0	{ 1	+ 1 day
	{ 2	+ 1 day		{ 2	+ 1 day		{ 2	+ 1 day
0·4	{ 3	+ 1 day	2·0	{ 3	+ 1 day	3·5	{ 3	+ 2 days
	{ 4	+ 2 days		{ 4	+ 1 day		{ 4	+ 2 days
0·2	{ 5	+ 1 day	1·0	{ 5	+ 1 day			
	{ 6	+ 2 days		{ 6	+ 1 day			
0·1	{ 7	+ 2 days	0·6	{ 7	0			
	{ 8	0		{ 8	0			
			0·4	{ 9	0			
				{ 10	0			
			0·2	{ 11	0			
				{ 12	0			
			0·1	{ 13	0			
				{ 14	0			

TABLE XIX.

The original solution contained 5 mg. of nucleoprotein per cubic centimetre.

Age	Lethal dose *
Fresh	0·1 c.c.
4 days	0·4
17 days	about 4·0

* Portions removed from time to time to test toxicity were diluted 1 in 5.

TABLE XX.

Protocols of experiments in determination of toxicity, in Table XIII above.

Fresh solution.			Solution incubated for 4 days.			Solution incubated for 17 days.		
Dose, c.c.	Rat	Result	Dose, c.c.	Rat	Result	Dose, c.c.	Rat	Result
0·4	{ 1	+ 1 day	0·4	{ 1	+ 2 days	0·6	{ 1	+ 2 days
	{ 2	+ 1 day		{ 2	0		{ 2	0
0·3	{ 3	+ 1 day	0·3	{ 3	+ 3 days	0·4	{ 3	+ 2 days
	{ 4	+ 1 day		{ 4	0		{ 4	+ 2 days
0·2	{ 5	+ 1 day	0·2	{ 5	0	0·2	{ 5	0
	{ 6	+ 2 days		{ 6	0		{ 6	0
0·1	{ 7	+ 2 days	0·1	{ 7	0	0·1	{ 7	0
	{ 8	0		{ 8	0		{ 8	0
0·05	{ 9	0						
	{ 10	0						

The accurate determination of the lethal dose of toxin demands a large number of animals. The results obtained in these three cases are sufficient to establish the fact that on hydrolysis there is a progressive loss of toxicity but are not sufficient to effect correlation between the extent of the loss and the work done by the ferment.

2. *Effect on the immunising power.*

As in the previous series of experiments the hydrolytic agent was the enzyme naturally occurring in the extract containing the nucleoprotein. The extract contained 8 milligrammes of nucleoprotein per cubic centimetre, and possessed a faintly alkaline reaction to litmus paper. It was kept at a temperature of 37° C. The amount of hydrolysis taking place in such an extract so kept and its effect upon the toxicity of nucleoprotein have already been considered (*vide* pp. 28, 36 above). We have now to determine what is the effect of the hydrolysis of the solution upon its immunising property.

In the first report (*vide* this *Journal*, 1910, Vol. x. p. 559), it was shown that a dose of extract containing 0.01 mg. nucleoprotein immunised from 80–90 % of rats against infection by a standard dose of the virulent living culture.

The source of this extract was an emulsion of organisms killed by chloroform vapour. We now know that during the time of exposure to chloroform, hydrolysis is proceeding, for it has been shown that it takes place in a chloroform water suspension. In dealing with the relation between hydrolysis and immunising power, it therefore follows that account must be taken of the influence of the time factor in the method of preparation.

As a preliminary an extract was prepared according to the original method which may be considered to have been hydrolysing for one day. The results of its inoculation into rats confirmed those obtained a year before, that a volume containing 0.01 mg. nucleoprotein protects 80–90 % the vaccinated animals.

TABLE XXI.

Protection afforded by extract hydrolysed for one day.

Dose, mg.	No. of rats inoculated	No. survived	Percentage surviving
0.01	19	17	89

Preliminary trials having indicated that the effect of hydrolysis on the immunising properties of the extract was small it was allowed to autolyse at 37°C. for upwards of two months. After one month 14 rats were inoculated with a volume which contained 0.01 mg. of nucleoprotein at the beginning of this lengthy incubation. Of these animals 12 survived the subsequent test dose, *i.e.* 83 %. It follows then that the lysis of the nucleoprotein far from having destroyed its immunising power, had left it unaltered. Referring to what has already been said in this report as to the effect of lysis, it will be remembered that at this stage of the process some half of the available nitrogen has been transformed to a condition not precipitable by tannic acid mixture.

Having then failed to find that after one month's autolysis there was any diminution in the immunity conferred by the extract, corresponding to the reduction of toxicity, it was returned to the incubator and hydrolysis allowed to continue for a further period of six weeks. At the end of this time 15 rats received the same dose as before and of these 11 survived the subsequent standard infecting dose of living culture, giving a protection of 73 %.

The diminished percentage of protections may indicate that at this late stage of hydrolysis the immunising value of antigen is beginning to be affected, but in a series of 15 rats it is hardly outside the statistical error.

We are thus obliged to admit that the protection afforded by a solution of the nucleoprotein that was originally toxic is but slightly, if at all, diminished by allowing the solution to hydrolyse whereas this process has been shown to destroy the toxicity of the solution.

In addition to the experiments just mentioned where after one month and two and a half months the animals received of the autolysed extract doses whose volumes corresponded to 0.01 mg. of the original nucleoprotein content, other series of rats were inoculated with one-tenth this quantity of extract. Thus of 15 rats that received the dose equivalent to 0.001 mg. of the one month's hydrolysed extract, eight survived the subsequent test inoculation (53 %), and of 15 rats that received the dose equivalent to 0.001 mg. of the two and a half months' hydrolysed extract seven survived the test inoculation (46 %). Reducing the dose of the one month's hydrolysed extract to one-tenth thus reduced the percentage protection from 86 to 53 and reducing the dose of the two and a half months' hydrolysed extract to the same amount reduced the percentage protection from 73 to 46.

In both cases the same reduction of the dose effected approximately the same reduction in the percentage protection.

This result affords corroborative evidence as to the relative accuracy of the percentages given by the experiment and used as a measure of the amount of immunising substance in the extract.

Putting these results in the form of a table we have :

TABLE XXII.

Effect of hydrolysis on immunising substance.

Time of hydrolysis	Dose, mg. nucleoprotein	No. of rats inoculated	No. of rats surviving	Percentage surviving
1 day	0.01	19	17	89
1 month	0.01	14	12	83
	0.001	15	8	53
2½ months	0.01	15	11	73
	0.001	15	7	46

The effect of hydrolysis under the influence of the hydrolytic enzyme normally present in extracts of the plague bacillus upon the immunising power presents analogies with Ruffer and Wilmore's (1908) results upon the hydrolysis of the endotoxin of the dysentery bacillus¹. These authors found that by digesting for three days with pepsin and hydrochloric acid the toxicity was reduced to $\frac{1}{30}$ th whilst the immunising value was not greatly, if at all, diminished. Gottstein (1908) also digested an emulsion of typhoid bacilli with pepsin and obtained a soluble product which he called "fermo toxin." This solution was toxic for guinea pigs and possessed of immunising action which according to this author was not impaired by boiling².

V. THE INCREASE OF IMMUNISING POWER AS THE RESULT OF HYDROLYSIS.

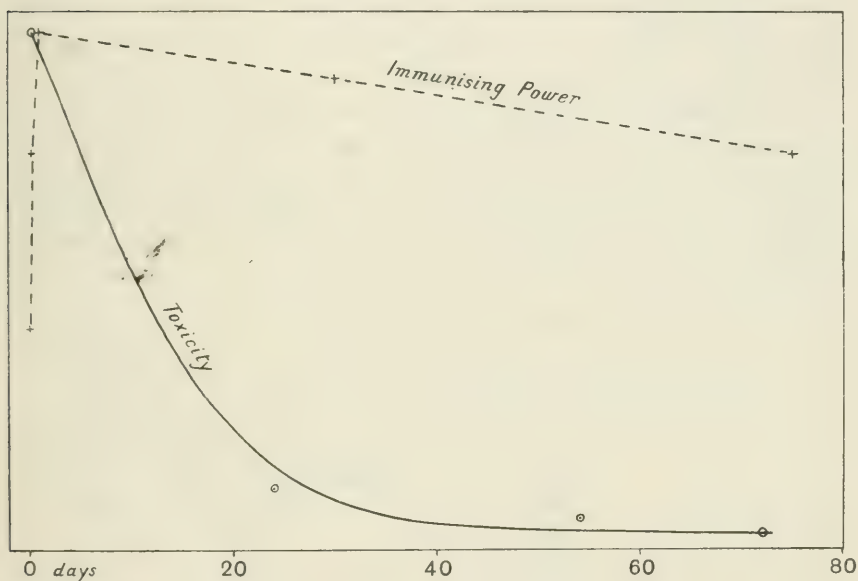
The extract prepared by the method detailed in the last report has been considered as having been subjected to hydrolysis during the time occupied in the preparation. Roughly we may consider such an extract to have been hydrolysed for one day. When it is remembered that the rate of hydrolysis is greatest in the earlier stages, the importance of being able to compare the effect upon the toxicity and immunising value in these early stages becomes obvious.

¹ *British Medical Journal*, 1908, Vol. II. p. 1176.

² *Deutsches Arch. f. klin. Med.* Vol. xciv. p. 255.

In the method as originally described the bacilli grown on the surface of agar in Roux flasks were submitted to the action of chloroform vapour for 12 hours; during the whole of this time, as we now know, hydrolysis was proceeding. If this time could be reduced or abolished we should be in the position of obtaining extracts which had been hydrolysed for only the reduced time or not at all.

An extract was therefore prepared so that the time of exposure of the organisms from which it was prepared—and so the time of hydrolysis—was reduced to *two* hours.



Of 33 rats inoculated with such a volume of this extract as contained 0.01 mg. nucleoprotein, 24 survived the subsequent standard infecting dose of virulent culture, = 73 %. This degree of protection is less than that obtained by a similar dose of what we have called the one day's hydrolysis extract (80–90 %), but taken by itself would hardly be significant.

To still further reduce the time of hydrolysis, an extract was prepared in which the use of chloroform as a killing agent was dispensed with, the bacilli being scraped off the surface of the agar

alive, washed alive, and mixed alive with the anhydrous sulphate of soda, which killed them¹.

The only opportunity for hydrolysis to occur is in the final extraction of the sulphated organisms. This process occupied a short time only and took place at the temperature of the laboratory. The extract prepared from this material is therefore hydrolysed to a minimum possible under the circumstances.

Of 42 rats inoculated with a volume of this extract which contained 0.01 mg. of nucleoprotein, only 21 or 50% survived the subsequent standard injection. This experiment is distinctly significant and indicates that the initial effect of hydrolysis is to *increase* the protection afforded by the same dose of nucleoprotein, and that a well hydrolysed extract is a better vaccine than one that has been submitted to the action of the enzyme to a less degree.

If we now put in the form of a table the figures representing the protection afforded by the various extracts (percentage rats surviving the standard infecting dose) and the figures expressing the hydrolysis time, it becomes clear that so far from hydrolysis diminishing the protective value, in the early stages of the process it actually increases it.

TABLE XXIII.

Relation between the protective value of the extracts and the amount of hydrolysis.

Hydrolysis time	Percentage rats protected by amount of extract originally containing 0.01 nucleoprotein
Fresh	50
2 hours	73
1 day	89
1 month	83
2½ months	73

From the above experiments the very important conclusion may be drawn that *the protective and toxic properties of the extract are distinct*. These may belong either to two different substances which are difficult to separate or be two properties attached to the same substance. So far neither has been observed apart from the nucleoprotein which they accompany throughout the various manipulations to which this substance

¹ As long as the bacilli are manipulated alive their contents do not escape into the solution. This is however a dangerous process and to anyone who wishes to repeat it the precaution of performing the mixture in an efficient draught cupboard is advised. During the final stages of the mixing the finely powdered material floats in clouds in a slight current of air.

has been subjected. That, under the hydrolytic influence of the enzyme, the toxicity is greatly diminished whereas the protective power only manifests itself more markedly, is explicable on the assumption that both activities are proper to the nucleoprotein. If such be the case, the effect of hydrolysis suggests that the toxicity is bound up with the basic protein moiety and the immunity-conferring property with the nuclein part, as the nuclein complex is known to be highly resistant to hydrolytic agencies.

VI. IMMUNITY AGAINST THE TOXIN.

Hitherto when referring to immunising properties the protective effect as discovered by a subsequent inoculation of living plague *only* has been considered. Since the anti-infectious antigen appears to be independent of the toxin it was legitimate to suspect that the immunity conferred upon my rats does not depend upon the development of antitoxin. Antitoxin, however, may be produced by the repeated injection in steadily increasing amount of the toxic nucleoprotein. This aspect of the subject is treated in another report (see this *Journal*, p. 11 above) but it is necessary to mention here that by treating a horse on much the same principle as is employed in the manufacture of diphtheria antitoxin a serum of quite encouraging antitoxic value was obtained.

The following experiments were made to determine whether any resistance to the toxic extract is conferred by a previous single injection of a sublethal dose of the toxic extract or of the atoxic product resulting from the autolysis of the same.

A series of rats were injected subcutaneously with doses varying from '00005 mg. to '05 mg. of toxic nucleoprotein. The highest dose is about half that quantity which usually kills a rat in 24 hours, and one-third of all the rats which received it died before the time for determining their resistance had arrived. Hence, those ultimately tested were to some extent selected rats. The extract was similar to that which repeated experience has shown will protect against a subsequent infection by the living organisms in doses of '001 to '01 (1910, p. 559).

The tests for resistance were made one month after the inoculation. In the first series of experiments the test dose varied from '2 to 3'5 average M.L.D.'s. The results are set forth in Table XXIV and show a considerable degree of resistance to have been developed by the injection of the largest dose.

TABLE XXIV.

Test of immunity conferred by a single dose of toxic nucleoproteid against a subsequent (one month) dose of toxic nucleoprotein.

Test doses M.L.D.'s	Rat	Immunising dose, .05	0.005	0.0005	0.00005 mg.
3.5	{ 1	++	+	+	+
	{ 2	0 0	0	+	+
3.0	{ 3	0 0	+	+	+
	{ 4	0 0	0	+	+
2.5	{ 5	0 0	+	+	+
	{ 6	0 0	+	0	+
2.0	{ 7	0 0	+	+	+
	{ 8	0 0	+	0	+
1.5	{ 9	0 0	0	+	+
	{ 10	0	0	+	+
1.0	{ 11	0	+	0	+
	{ 12	0	0	0	+
0.5	{ 13	0	0	0	+
	{ 14	0	0	0	0
0.2	{ 15	0	0	0	0
	{ 16	0	0	0	0

The next series of experiments was devised to ascertain whether any similar antitoxic immunity was aroused by the nucleoprotein extract after it had been mostly deprived of toxicity by hydrolysis (see p. 36 above). The toxicity of the hydrolysed material was found to be at most one-tenth of the original. It must be remembered that the atoxic extract protects equally well against infection by the living organisms.

Table XXV below, in which the resistance developed with toxic and atoxic extracts is compared, shows that the large dose .05 mg. of either confers a considerable degree of immunity, and from this experiment alone it is not possible to conclude that the toxic preparation possessed any advantage.

TABLE XXV.

Immunity against toxin by toxic and atoxic nucleoprotein respectively.

Immunising dose, mg...	0.05		0.005		0.0005	
	Toxic	Atoxic	Toxic	Atoxic	Toxic	Atoxic
Test dose	{ Died 2	Died 2	Died 2	Died 7	Died 4	Died 7
3 M.L.D.'s	{ Survived 6	Survived 6	Survived 2	Survived 1	Survived 0	Survived 1
Test dose	{ Died 0	Died 2	Died 4	Died 3	Died 4	Died 6
2 M.L.D.'s	{ Survived 11	Survived 5	Survived 2	Survived 5	Survived 2	Survived 2
Total survived	89 %	73 %	40 %	37 %	20 %	19 %

Controls 22 %.

This result is contrary to expectation, and the matter is receiving further attention.

The conclusion that the toxicity and protective property of the extract are independent finds support from the results of experiments upon the protective value of the toxic nucleoprotein to which an anti-serum (*supra* p. 11) had been added.

A series of rats was inoculated with the serum alone, the toxic nucleoprotein alone, and with varying quantities of a neutralised mixture of the two. Altogether six series were prepared and two weeks later the degree of immunity developed by the animals was tested by the inoculation of the standard dose of the living virulent culture. The results are set forth in Table XXVI below :

TABLE XXVI.

EXP. 1. One-tenth lethal dose toxic nucleoprotein and serum to correspond.

	No. rats inoculated	No. protected	Percentage protected against subsequent infection
Serum alone	14	7	50
Toxic nucleoprotein alone	19	15	79
Mixture	15	12	80

EXP. 2. 10 neutralised lethal doses.

10	9	90
----	---	----

EXP. 3. 20 neutralised lethal doses.

8	8	100
---	---	-----

EXP. 4. 50 neutralised lethal doses.

9	8	90
---	---	----

It will be seen from these results that the serum neutralised the toxicity without apparently affecting the substance to which protection is due. Exp. 1 in which the dose of nucleoprotein was only just optimal shows that the neutralisation of the toxicity has had no effect on the protective value of the solution. Thus the toxin may be put out of action either by neutralisation or by hydrolysis. In either case the particular antigen which is responsible for protection is, or becomes, free to act.

Working as a member of a large staff of an institute with many-sided activities, it is difficult to express individual thanks to the many of my colleagues who have helped me in the course of this investigation. I must however express my sense of obligation to Dr C. J. Martin, Director of the Lister Institute, whose constant advice and service have always been at my disposal.

VII. CONCLUSIONS.

1. The toxic and immunising properties of the plague bacillus are attached to nucleoproteins which are soluble in water and can be extracted from the bacilli by suitable means.

2. Chloroform forms compounds with these nucleoproteins which are reversible.

3. These compounds with chloroform are relatively non-toxic and possess greatly diminished immunising powers against infection with the living organism but on removal of the chloroform the toxicity and immunising properties are regained.

4. Toluol forms similar compounds which behave in a similar manner to the analogous compounds with chloroform.

5. The effect of heat on the toxic and immunising powers of the nucleoproteins whilst in the bacillus is much less than on the toxic and immunising powers of the nucleoproteins after extraction from the bacillus. Solutions of nucleoproteins of full immunising and toxic power can be obtained from bacilli that have been heated to 60° C., whereas these properties are destroyed if solutions of the nucleoproteins exhibiting them which have been extracted from the bacilli are heated to this temperature.

6. The plague bacillus contains a proteolytic enzyme.

7. The extracts containing nucleoproteins in solution obtained under circumstances precluding as far as possible the action of this enzyme are more toxic than similar extracts obtained under circumstances allowing the enzyme to act.

8. In the early stages of the action of the enzyme on extracts containing nucleoproteins in solution exhibiting toxic and immunising powers, a fall in the value of the toxic power accompanied by a rise in the value of the immunising power occurs; by continued hydrolysis the toxicity practically disappears but the immunising power is not diminished.

9. Experiments show that some resistance against toxic extracts, as well as against infection, may be conferred by the inoculation of large doses of the same toxic extracts either in their original condition or in the atoxic modification produced by hydrolysis.

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No 56

XLII. PRELIMINARY OBSERVATIONS ON THE PROTECTIVE AND CURATIVE VALUE FOR RATS OF THE SERUM OF A HORSE IMMUNISED WITH A TOXIC NUCLEO-PROTEIN EXTRACTED FROM THE PLAGUE BACILLUS.

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IN a previous report (Vol. x. p. 536) I have dealt with a method of extracting from the plague bacillus a substance or substances which appear to be responsible both for the acute toxic action and immunising properties of killed plague bacilli. I have since investigated the properties of the serum of a horse immunised with this solution. In this inquiry I have had the advantage of the assistance of Dr A. T. MacConkey, Head of the Serum Department of the Lister Institute, who kindly undertook the supervision of the immunisation of the horse and to whom I desire to express my thanks.

For the purposes of the experiment a horse was selected and immunisation commenced on June 26, 1909. The material injected into the horse consisted of nucleo-protein dissolved in dilute saline prepared as explained in my previous report, where it was designated solution B. The method of immunisation adopted was as far as possible that usually employed in the Serum Department of the Lister Institute for the preparation of diphtheria antitoxin. The table gives the dates and doses administered intramuscularly. Unfortunately owing to lack of toxin the intervals towards the end of immunisation were longer than were absolutely necessary.

The local reaction after inoculation was almost identical with that observed after inoculation of diphtheria toxin. Some tenderness was present and swelling and oedema varying in amount, with transitory

constitutional disturbance and little temperature reaction. There was no tendency to abscess formation or to the huge hard swellings of long duration which are not infrequently the result of injection of unfiltered cultures.

TABLE.

Date	Dose in mgs.	Date	Dose in mgs.
June 26	0.01	July 26	28.0
28	0.02	Aug. 3	42.0
July 3	0.1	10	80.0
7	0.2	24	100.0 Sample bleeding
9	0.4	Sept. 9	15.0 taken.
13	0.8	Oct. 26	30.0
15	1.6	Nov. 13	60.0
17	1.6	20	120.0
20	3.5	Dec. 8	240.0
22	7.0	29	Bled 8 litres.
24	14.0		

Neutralisation of the toxic extract (solution B) by means of the serum.

The first point to settle was whether the serum contained any substance capable of neutralising the toxin contained in solution *B*, *i.e.*, the toxin with which the horse had been immunised.

Method. The method used to investigate this point was to mix a known number of lethal doses of the toxin (M.L.D. = 0.1 mg. nucleo-protein) with a known volume of the serum, to leave the mixture at 35° C. for 30 minutes and then to inject it subcutaneously into rats and observe the result. If the rat lived, it was considered that the toxin had been neutralised.

Experiment 1¹.

Rat	No. of L.D. of toxin	Serum in c.c.	Result
1)	10	0.1	—
2)			—
3)	25	0.1	+ 1 day
4)			+ 1 day

1/10th c.c. of serum neutralises 10 lethal doses of toxin but not 25.

¹ Throughout these records — or 0 = lived, + = died.

Experiment 2.

Rat	No. of L. D. of toxin	Serum in c.c.	Result
1)	5.0	0.1	-
2)			-
3)			-
4)	7.5	0.1	-
5)			-
6)			-
7)	10.0	0.1	-
8)			+ 1 day
9)			+ 1 day
10)	12.5	0.1	+ 1 day
11)			+ 2 days
12)			+ 2 days
13)	15.0	0.1	+ 1 day
14)			+ 1 day
15)			+ 1 day
16)	17.5	0.1	+ 1 day
17)			+ 1 day
18)			+ 1 day

The serum used in this experiment was from a second bleeding 5 days after the first bleeding. 1/10th c.c. of this serum neutralised 7.5 lethal doses of toxin.

In these experiments (1 and 2) the dose of serum was kept constant and the dose of toxin varied; in the next series the dose of toxin was kept constant and the dose of serum varied.

Experiment 3. Estimation of the amount of serum required to neutralise 10 lethal doses of toxin B.

Rat	Serum in c.c.	Result
1)	0.08	+ 1 day
2)		+ 1 day
3)		+ 1 day
4)		-
5)		-
6)	0.1	+ 1 day
7)		+ 1 day
8)		+ 1 day
9)		-
10)		-
11)	0.12	-
12)		-
13)		-
14)		-
15)		-
16)	0.14	-
17)		-
18)		-
19)		-
20)		-

10 lethal doses toxin were neutralised (*qua* rat) every time by 0.12 c.c. serum and four out of ten times by 0.1 and 0.08 c.c. serum.

It having been shown that the amount of serum necessary to neutralise 10 lethal doses of the toxin, when mixed with it before injection into a rat, lies between 0.1 and 0.12 c.c., we have now to determine whether this ratio still holds when dealing with a greater number of lethal doses of the toxin. For this purpose 100 lethal doses of toxin were mixed with the quantities of serum shown in the following table: the mixture stood for one hour at 37° C. and was then injected into rats.

Experiment 4.

Rat	No. of L.D. of toxin	Serum in c.c.	Result
1	100	2	Survived
2			
3		1.75	,,
4			
5		1.5	,,
6			
7		1.25	,,
8			

From this experiment it is evident that the value of the ratio is maintained.

We conclude then *that the serum of this horse can neutralise the toxin in solution B.*

As affording some information on the progress of immunisation we give here the result of a test made, about half-way through the immunisation process, of a sample bleeding on August 24th.

Experiment 5.

Rat	L.D. toxin	Serum in c.c.	Result
1	102	1.0	+ 1 day
2			+ 1 day
3	68	1.0	+ 2 days
4			+ 3 days
5	34	1.0	—
6			—
7	3.4	1.0	—
8			—

At this time 1 c.c. of serum neutralised 34 lethal doses of toxin, compared with between 85 and 100 at the time of the final bleeding.

Effect of Yersin serum on the toxin in solution B.

It is necessary to compare with this serum that obtained by Yersin's method of immunisation, *i.e.* the ordinary anti-plague serum on the market, which is prepared by the inoculation of dead, and later of living, cultures of the plague bacillus.

Experiment 6. Estimation of amount of Yersin serum (prepared at the Lister Institute) necessary to neutralise 10 lethal doses of toxin.

Rat	Serum in c.c.	Result
1	2.0	+ 1 day
2		+ "
3		+ "
4		+ "
5		+ "
6	1.0	+ "
7		+ "
8		+ "
9		+ "
10		+ "
11	0.5	+ "
12		+ "
13		+ "
14		+ "
15		+ "
16	0.1	+ "
17		+ "
18		+ "
19		+ "
20		+ "

No evidence of any antitoxic action is shown by this serum.

Similar results were obtained with two other samples of Yersin serum (Négress and Mayboy).

In the case of two other samples (Kit and Corbally) 0.5 c.c. of the serum neutralised 10 lethal doses of toxin and 0.25 c.c. serum failed to do so.

Prophylactic value of the serum against infection.

The serum of the horse immunised with solution *B* is capable, in common with some of the sera obtained by the usual methods of immunisation, of protecting against a subsequent inoculation of virulent plague. In experiment 7 (p. 16), the rats received the amount of serum indicated, and the standard test dose of virulent culture was given on the following day.

Curative value in plague infection.

So far attention has been confined to the antitoxic and anti-infectious properties of the serum, and the question remaining to be answered is:—has the serum any effect when administered to an animal that is already suffering from plague?

Experiment 7.

Dose of serum	Rat	Result
1·0 c.c.	1	0
	2	0
	3	0
	4	0
	5	0
	6	0
	7	0
	8	0
	9	0
	10	0
0·1 c.c.	11	+ 4 days
	12	0
	13	0
	14	0
	15	0
	16	0
	17	0
	18	+ 3 days
	19	0
	20	+ 4 days
0·01 c.c.	21	+
	22	+
	23	+
	24	+
	25	+
	26	+
	27	0
	28	+
	29	+
	30	0

For this purpose the following experiment was performed. Sixty rats were inoculated with the usual dose of a virulent living plague culture: ten of these were left as controls, of which seven died of plague. The remaining fifty were treated in batches of ten—five batches. Six hours after inoculation with the living culture, batches 2, 3, 4, 5 and 6 received 0·5 c.c. serum subcutaneously on the opposite side of the body to that on which the living plague culture had been inoculated. Six hours later batches 3, 4, 5, and 6 received a second and similar dose; six hours later batches 4, 5, and 6 a third; six hours later batches 5 and 6 a fourth, and six hours later batch 6 received a fifth dose.

The results of this treatment are shown in experiment 8.

This table shows:

(1) That the infectivity of the test virulent culture was well up to the standard (Vol. x. p. 544).

(2) That taking all the rats together the death rate had been reduced from 80% to 18%.

Experiment 8.

		Treatment after infection.					Serum c.c.		
Batch	Rat	6 hrs.	12 hrs.	18 hrs.	24 hrs.	30 hrs.	Result	Average	
1	1	...	...	...	...	...	+ 3 days	3.5 days	
	2	...	...	...	...	...	+ 3 days		
	3	...	...	...	...	...	+ 3 days		
	4	...	...	...	...	...	+ 4 days		
	5	...	Controls.	No treatment.	...	...	+ 4 days		
	6	...	...	...	...	...	+ 4 days		
	7	...	...	...	...	...	+ 4 days		
	8	...	...	...	...	...	0		
	9	...	...	...	...	...	0		
	10	...	...	...	...	...	0		
2	1	0.5	...	...	...	...	+ 4 days	5 days	
	2	0.5	...	...	...	...	+ 6 days		
	3	0.5	...	...	...	...	0		
	4	0.5	...	...	...	...	0		
	5	0.5	...	...	...	...	0		
	6	0.5	...	...	...	...	0		
	7	0.5	...	...	...	...	0		
	8	0.5	...	...	...	...	0		
	9	0.5	...	...	...	...	0		
	10	0.5	...	...	...	...	0		
3	1	0.5	0.5	...	...	...	+ 9 days	6½ days	
	2	0.5	0.5	...	...	...	+ 4 days		
	3	0.5	0.5	...	...	...	0		
	4	0.5	0.5	...	...	...	0		
	5	0.5	0.5	...	...	...	0		
	6	0.5	0.5	...	...	...	0		
	7	0.5	0.5	...	...	...	0		
	8	0.5	0.5	...	...	...	0		
	9	0.5	0.5	...	...	...	0		
	10	0.5	0.5	...	...	...	0		
4	1	0.5	0.5	0.5	...	...	0		
	2	0.5	0.5	0.5	...	...	0		
	3	0.5	0.5	0.5	...	...	0		
	4	0.5	0.5	0.5	...	...	0		
	5	0.5	0.5	0.5	...	...	0		
	6	0.5	0.5	0.5	...	...	0		
	7	0.5	0.5	0.5	...	...	0		
	8	0.5	0.5	0.5	...	...	0		
	9	0.5	0.5	0.5	...	...	0		
	10	0.5	0.5	0.5	...	...	0		
5	1	0.5	0.5	0.5	0.5	...	+ 6 days	6 days	
	2	0.5	0.5	0.5	0.5	...	+ 6 days		
	3	0.5	0.5	0.5	0.5	...	0		
	4	0.5	0.5	0.5	0.5	...	0		
	5	0.5	0.5	0.5	0.5	...	0		
	6	0.5	0.5	0.5	0.5	...	0		
	7	0.5	0.5	0.5	0.5	...	0		
	8	0.5	0.5	0.5	0.5	...	0		
	9	0.5	0.5	0.5	0.5	...	0		
	10	0.5	0.5	0.5	0.5	...	0		
6	1	0.5	0.5	0.5	0.5	0.5	+ 9 days	10 days	
	2	0.5	0.5	0.5	0.5	0.5	+ 10 days		
	3	0.5	0.5	0.5	0.5	0.5	+ 11 days		
	4	0.5	0.5	0.5	0.5	0.5	0		
	5	0.5	0.5	0.5	0.5	0.5	0		
	6	0.5	0.5	0.5	0.5	0.5	0		
	7	0.5	0.5	0.5	0.5	0.5	0		
	8	0.5	0.5	0.5	0.5	0.5	0		
	9	0.5	0.5	0.5	0.5	0.5	0		
	10	0.5	0.5	0.5	0.5	0.5	0		
50 treated								9 deaths	

(3) That in those cases where death of the treated rats occurred the length of life after injection was prolonged from 3-5 days to 10 days.

The next experiment was to test the maximum length of time that could be allowed to elapse between infection with plague and the inoculation of serum, consistent with the survival of the animal.

For this experiment 30 rats received the standard dose of living virulent culture. The rats were divided into three batches of 10 each. Batch 1 were not treated and remained as controls. Batch 2 received 0.5 c.c. serum 24 hours after infection. Batch 3 received 0.5 c.c. serum 48 hours after infection. The results of this experiment are shown in table 9.

Experiment 9.

Batch	Rat	Treatment	Days after infection										
			1	2	3	4	5	6	7	8	9	10	
2	1	0.5 c.c. serum 24 hours after infection	...	...	...	...	...	...	...	...	...	...	Survived.
	2		...	...	...	...	...	...	...	...	...	...	Survived.
	3		...	...	...	...	...	...	...	...	...	...	Survived.
	4		...	...	...	...	...	...	...	...	...	...	Survived.
	5		...	...	...	...	...	...	...	...	...	...	Survived.
	6		...	...	...	...	...	...	...	...	...	...	Survived.
	7		...	...	...	...	...	...	...	...	...	...	Survived.
	8		...	...	...	...	...	...	...	...	...	...	Survived.
	9		...	...	...	...	+	...	...	...	...	...	Survived.
	10		...	...	...	+	...	...	...	...	...	...	Survived.
3	1	0.5 c.c. serum 48 hours after infection	...	...	+	...	...	...	...	...	...	...	Survived.
	2		...	...	...	...	...	...	...	...	...	...	Survived.
	3		...	...	+	...	...	...	...	...	...	...	Survived.
	4		...	...	...	...	...	...	...	...	...	...	Survived.
	5		...	...	+	...	...	...	...	...	...	...	Survived.
	6		...	...	+	...	...	...	...	...	...	...	Survived.
	7		...	+	...	...	...	...	...	...	...	...	Survived.
	8		...	...	...	...	...	...	...	...	...	...	Survived.
	9		...	...	...	...	...	...	...	...	...	...	Survived.
	10		...	...	...	...	...	...	...	...	...	...	Survived.

The serum therefore saves the lives of a sensible proportion of infected rats even if its administration is delayed for as long as 24 hours after inoculation. It should be noted that the untreated rats begin to die on the second day.

In the case of the untreated batch, all the rats died; this must be taken as indicating that the virulence of the test culture was well up to the standard (80% deaths) and does not indicate any increase in virulence of the culture or diminution in resistance of the rats.

A comparative test was made between the curative value of Yersin's serum as prepared by the Lister Institute and the serum prepared by inoculation of solution B. In both cases ten rats received, 6 hours

after inoculation, 0.5 c.c. of serum hypodermically on the opposite side to the infecting dose of virulent culture. Ten control untreated rats showed the virulence of the test culture to be up to the standard.

Experiment 10.

Batch	Rat	Treatment	Result. Days after infection									
			1	2	3	4	5	6	7	8	9	10
1	1	0.5 c.c. Yersin serum 6 hours after infection	...	...	...	+						
	2		...	...	...	...	+					
	3		...	...	...	+						
	4		...	...	...	...	+					
	5		...	...	...	...	+					
	6		...	...	...	...	...	...	...	...	...	Survived.
	7		...	...	...	...	+					
	8		...	...	...	...	...	...	...	...	...	Survived.
	9		...	...	...	...	+					
	10		...	...	...	...	+					
2	1	0.5 c.c. new serum 6 hours after infection	...	...	...	...	+					
	2		...	...	...	...	...	...	...	...	...	Survived.
	3		...	...	...	...	...	...	...	...	...	Survived.
	4		...	...	...	...	...	...	...	...	...	Survived.
	5		...	...	...	...	...	...	...	...	...	Survived.
	6		...	...	...	...	...	...	...	...	...	Survived.
	7		...	...	...	...	...	...	...	...	...	Survived.
	8		...	...	...	...	...	...	...	...	...	Survived.
	9		...	...	...	...	...	...	...	...	...	Survived.
	10		...	...	...	...	...	+				

It has thus been demonstrated that the *antiplague serum* prepared by immunising horses with a solution of the nucleo-protein from the plague bacillus prepared as described in the previous report possesses not only antitoxic and anti-infectious properties, but that it also exercises in rats a markedly curative effect when injected 24 hours after the animals have been infected with virulent plague bacilli.

These results are most encouraging and further research is being directed towards obtaining a still more powerful serum.

SOME NOTES ON INDOLE-REACTION AND ALLIED PHENOMENA.

BY HARALD SEIDELIN, M.D.
AND FREDERICK C. LEWIS, F.C.S.

(From the *Lister Institute, London, and the Thompson Yates Laboratories, University of Liverpool.*)

(With 6 Charts.)

PART I.

By H. SEIDELIN.

A SHORT time ago I (1911) described a method for the quantitative estimation of indole. It may be of interest to reproduce the curves obtained by the use of this method, in the study of several strains of *Bacterium coli*, as curves representing indole-production have seldom been published.

The curves given by Marshall (1907) are based upon a small number of estimations, none of which was made later than the 23rd day, if we consider *B. coli* only; maxima were found, in the case of this bacterium, on the 5th and the 14th day, respectively. The estimations are much more quickly carried out according to my technique than by the method employed by Marshall; I was therefore able to make their number comparatively large.

For the purpose of studying the variations in the quantity of indole, a large number of peptone-water tubes were prepared at the same time, each tube containing 10 c.c. of 1% peptone (Witte) solution with $\frac{1}{2}$ % of NaCl. Some of the estimations have however been made in 2% peptone-water tubes, but the differences are of no importance, as the estimations in question were made after several weeks, whilst noticeable differences, depending on the strength of the peptone solution, were

in various tests found present during the first few days only. The tubes were inoculated with one loop each of a peptone-water culture of the corresponding strain and incubated at 37° C. Subsequently one tube was taken out every day and the quantity of indole determined. In this way the quantity of liquid in the tube diminishes, of course, by evaporation from day to day, and it is quite possible that this

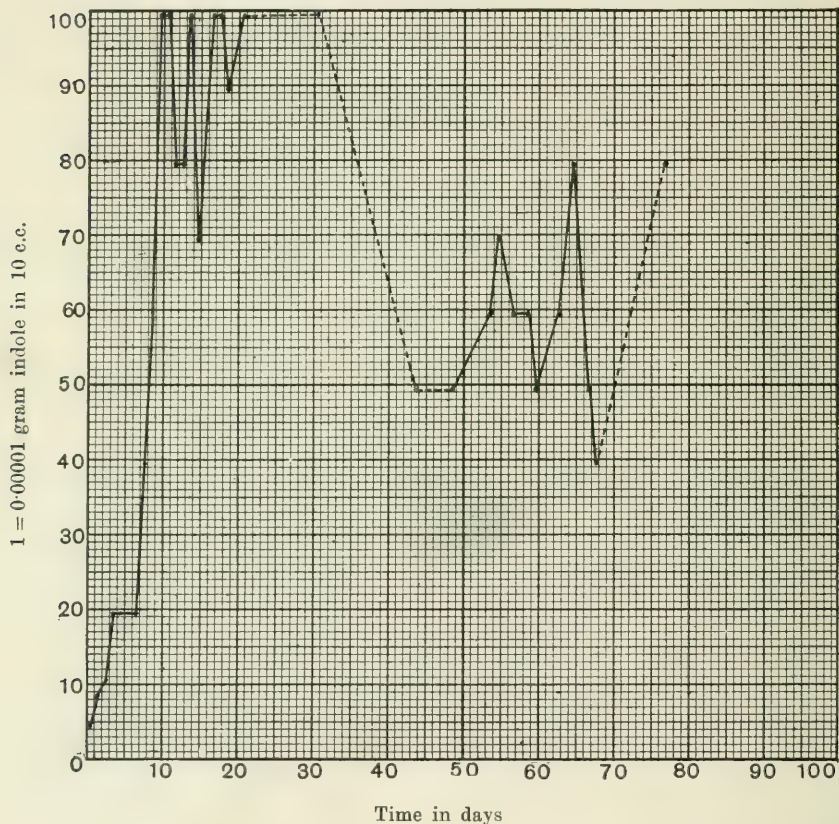


Chart I. *B. coli* Esch. a.

circumstance may influence the reactions, especially after two or three months when the quantity is much reduced. I do not think, however, that it would be advisable to proceed in any other way, as strong objections can be made against the two other procedures which suggest themselves, namely air-tight closing of the tubes, and the use of a flask culture, from which the quantity necessary for the test could be taken

daily. Air-tight closing would have the inconvenience that the reaction might proceed possibly in a different manner, when the free access of oxygen was excluded. If a flask culture was used, it would not be possible to obtain results of a uniform value; by abstracting from the culture 10 c.c. on, for instance, the 50th day, this amount would represent a far larger quantity of the original culture, which by then

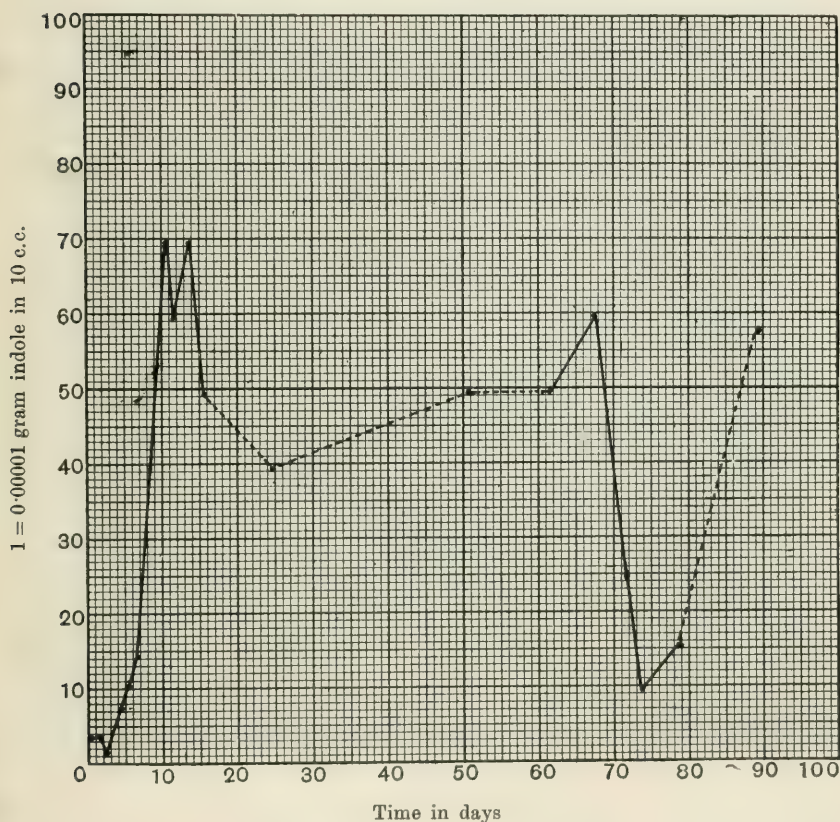


Chart II. *B. coli* (Pig faeces I).

would have become concentrated by evaporation. Consequently no comparison would be possible.

The advantage of the procedure adopted is, that each tube always contains the essential elements corresponding to the same quantity, 10 c.c., of the original peptone-water, notwithstanding the evaporation

of the water. The same amounts of the reagents were always used and, more especially, the same quantity of chloroform for extraction.

For the appreciation of the results it may be necessary to state that the comparative colorimetric scale in use consists of a series of tubes, the first one corresponding to a quantity 0.00001 gram indole to 10 c.c. of water and the following tubes to multiples of this, No. 100 consequently

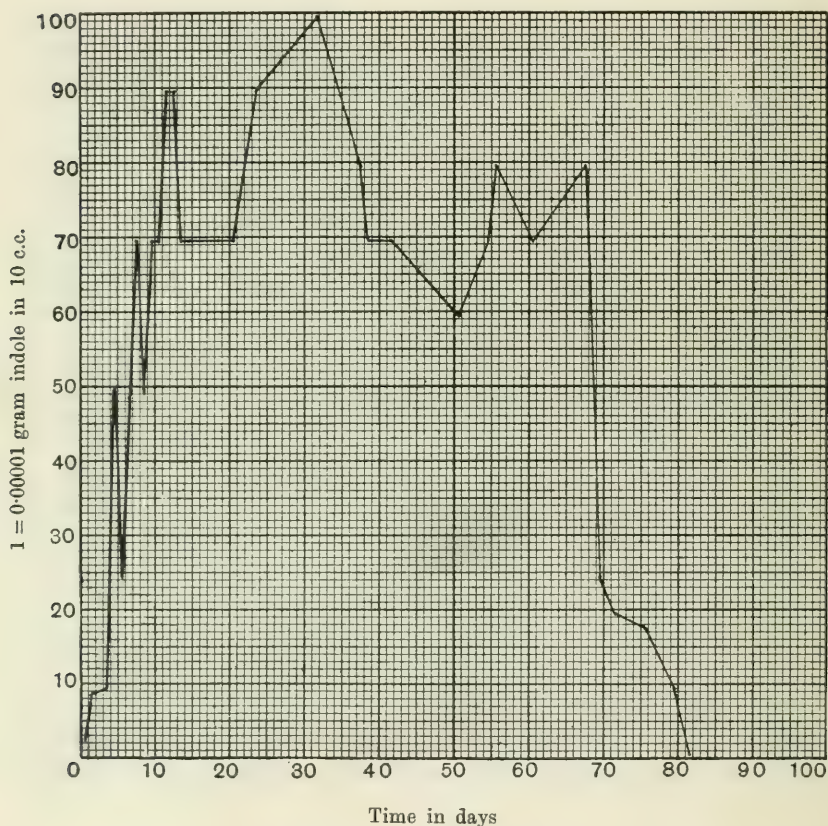


Chart III. *B. coli* (Pig faeces II).

to 0.001 gram in 10 c.c. For further detail the previous paper must be consulted.

The results obtained need no detailed discussion, as all particulars will become evident by a glance at the charts. All the strains examined were fairly strong indole producers; in all, with exception of *B. coli* (strain "Pig faeces I") represented in Chart II, a maximum of 100 or

just over, according to the scale, was obtained. The time, when the maximum was reached, differed considerably. The rise was in most cases gradual, although not quite regular and no remissions were seen before the maximum was reached. Afterwards, the curve lost its regularity and considerable oscillations occurred before a definite decrease began. The decrease was fully observed in two cases only (Charts III and V)

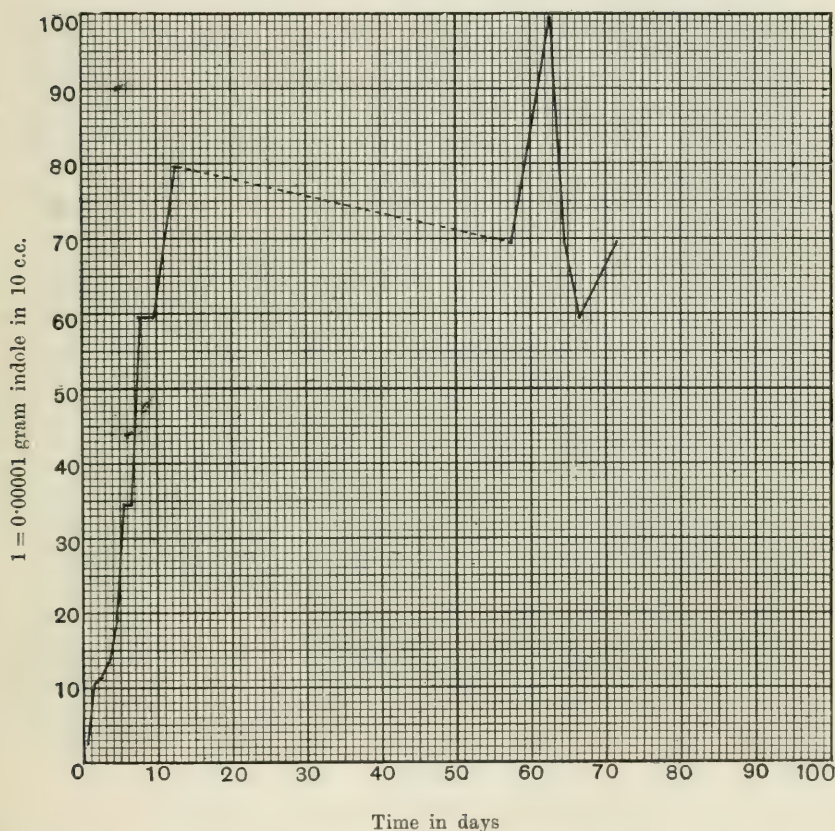


Chart IV. *B. coli* (Pig faeces III).

and in those the reaction disappeared between the 80th and 100th day.

The most interesting result is that indole was found present in the cultures, in considerable quantity, even when the tubes had been incubated for more than two months. Earlier authors, as Germano and Maurea (1893) and Marshall (1907) found a maximum of indole before

the end of the second week and did not follow the question up further.

So far, I cannot claim much value for the quantitative estimation of indole as a means of differentiating one strain of *B. coli* from another.

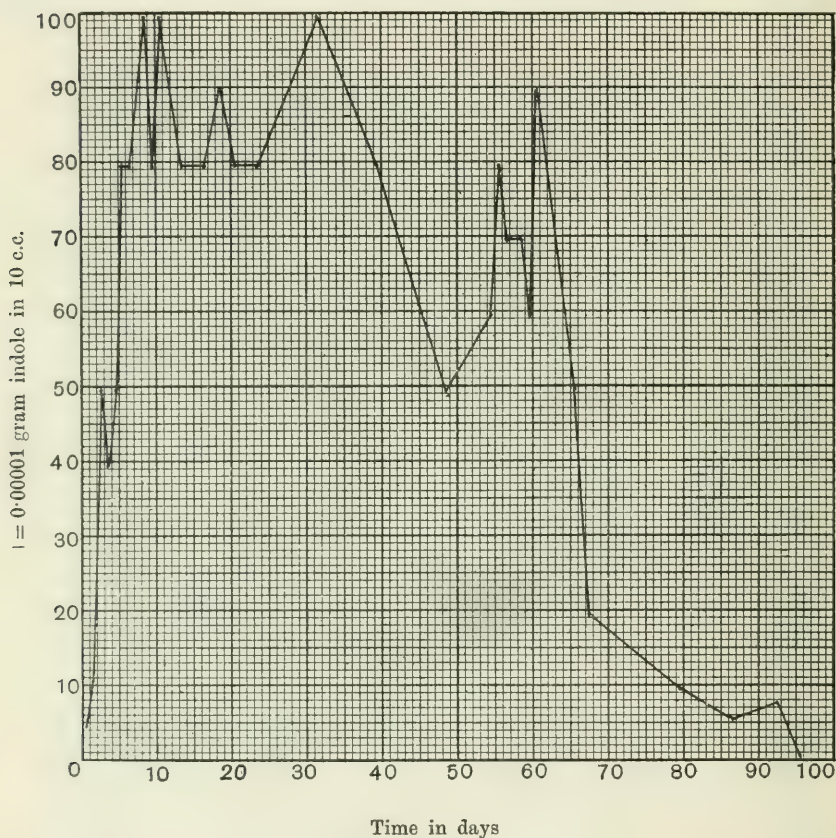


Chart V. *B. coli* Lab.

In a few cases it may be of use; in the strain represented in Chart II, for instance, the amounts of indole never approached those observed in the other strains. But in other cases the variations in cultures of the same strain were considerable. It is probable that the procedure has more interest in the study of the metabolism of bacteria.

In two of the strains a peculiar phenomenon was observed. When making the indole reaction in peptone-water cultures which had been

incubated for about eight days, a purple colour resulted instead of the ordinary red; by extraction with chloroform, only the red component passed into the extract, whilst the watery liquid took a blue colour, with the faintest possible trace of purple. Similar results were obtained when amyl-alcohol was used as the extracting liquid instead of chloroform.

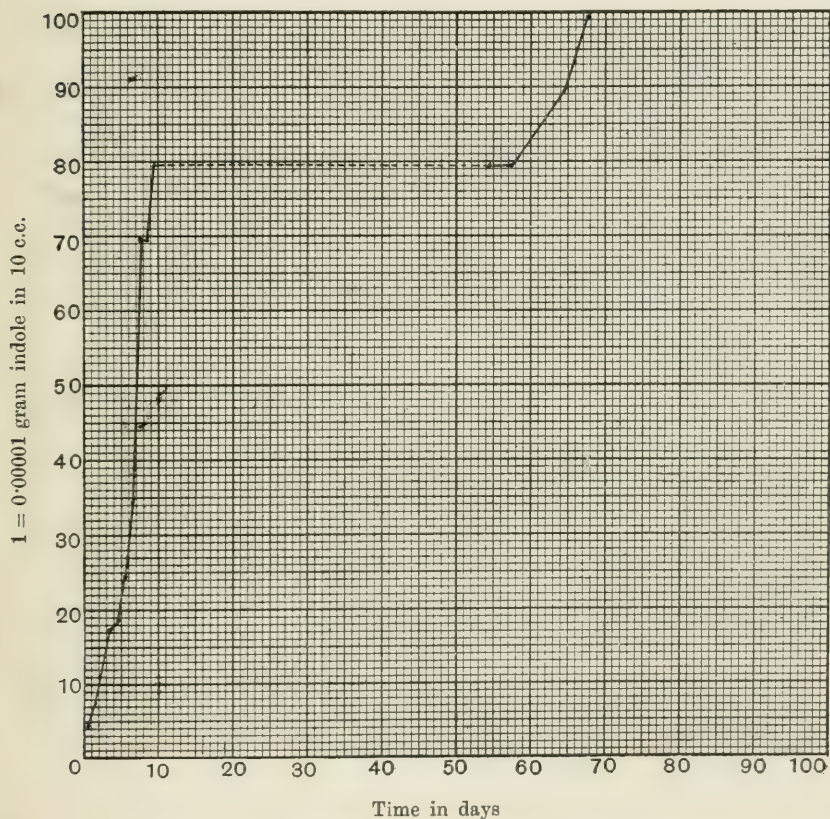


Chart VI. *B. coli* Esch. b.

The blue colour was first obtained in the course of the ordinary work with Ehrlich's reaction, executed in the manner described in my previous paper; afterwards a still more beautiful blue resulted by the use of a modified Steensma's (1906) reaction. The only difference between the two reactions, as I used them, was, that in Steensma's reaction a 0.5% sodium nitrite solution was substituted for the 1%

potassium persulphate in Ehrlich's, of the former only about ten drops being required.

Subsequently, similar reactions were observed with considerable frequency in the same two strains, but by no means constantly, nor always equally pronounced. It might even happen that several tubes, with identical quantities of peptone-water, inoculated at the same time and incubated for the same period gave quite different reactions. In one the colour after extraction might be quite blue, in another more purple, and in a third, almost red. All these coloured elements appeared, however, different from the ordinary red observed in the indole reaction, as even the red one could not be extracted by means of chloroform. The underlying causes of these differences are now being investigated.

The first idea, when a purple reaction was obtained, was, of course, that skatole might be present besides indole. That the blue colour was not due to skatole, became, however, evident when it failed to pass into chloroform and amyl-alcohol by extraction with these two substances, nor was the skatole reaction, with conc. sulphuric acid, according to the method of Sasaki (1910) obtained.

I was unable at that time to make further reactions, but Mr Lewis, to whom I demonstrated my results, soon found the same and similar phenomena in other cultures, in which he had already at an earlier date noticed a peculiar dark colour by Ehrlich's reaction, and undertook a further study of the substances concerned. A number of reactions were made, which Mr Lewis will communicate in the second part of this paper. We are now continuing these researches together and intend to publish the results at a later date.

Another interesting observation was made during my work with these bacteria. Ellinger and Gentzen (1904) found that the introduction of tryptophane into the coecum of rabbits gave rise to the excretion of a considerable amount of indican through the urine. The authors conclude that tryptophane represents a transitional stage in the formation of indole from proteins in the digestive tract.

This conclusion does not appear to be warranted by the experiments described; indole may well be formed from tryptophane when this substance happens to be present, but there is no proof that the process is a natural one. Still less justified of course would such a conclusion be concerning the rôle of tryptophane in the protein-decomposition by bacteria in general—an even more fundamental question. In order to throw some light on this question it seemed important to investigate

whether tryptophane could be demonstrated as a transitional product in cultures of indole-forming bacteria.

I therefore made the reaction for tryptophane with bromine water, as described by Abderhalden (1909), in peptone-water cultures of various strains, which were used in the other work mentioned in this paper. The reaction was made at different times, from a few hours to several days after the incubation of the cultures, both before my indole reaction had become apparent and when it was already quite strong. I never found the slightest indication of a positive result. That the reaction was not at fault, was proved by adding extremely small quantities of tryptophane to pure, uninoculated peptone-water and also to *B. coli*-cultures in peptone-water; a characteristic reaction was always obtained.

The result is therefore distinctly opposed to the conclusion drawn by Ellinger and Gentzen, but not to the result of their experiments. On the contrary, it is easy to demonstrate the formation of indole from tryptophane, by inoculating a tryptophane solution, with an indole-producing bacterium. I used a 0.5% tryptophane solution (with 0.5% NaCl) and obtained already on the following day a very strong indole reaction. The intensity of the indole reaction increased in the course of several days following and the tryptophane reaction, which was easily obtained even when the indole reaction had already become strong, disappeared in about a week.

Most of the work here detailed was executed at the Lister Institute, to the Director of which, Dr C. J. Martin, and to the Chief of the Bacteriological Department, Dr Ledingham, I feel greatly indebted.

PART II.

By FREDERICK C. LEWIS.

In applying Ehrlich's indole reaction I observed that in many cultures the colour produced was exceedingly pronounced, especially in those cases in which the tubes had been left standing for some hours. In such cases the colour was decidedly purple. Upon extraction with chloroform, as suggested by Dr Seidelin, I was able to confirm what he had already observed, namely the production of a blue colour in addition to the red due to presence of indole.

This phenomenon was then further studied and other points of interest demonstrated.

At least three reactions may take place other than the production of the characteristic red colour due to the presence of indole.

Group 1. The production of a "double red" colour.

Group 2. The production of a purple or blue colour in the presence of indole.

Group 3. The production of a blue or somewhat blue purple colour *in the absence of indole*.

The technique followed was according to the modifications proposed by Seidelin (1911):

After the incubation of a suitable culture 5 c.c. of the paradimethylamidobenzaldehyde reagent is added, followed by 5 c.c. of 1% potassium persulphate. The cultures are then left for a time and finally extracted with 5 c.c. of chloroform.

The length of time elapsing from the commencement of the reaction to the extraction with chloroform is a factor of great importance.

Group No. 1. (Production of the "double red" colour.)

An ordinary positive indole reaction is observed but, upon extraction with chloroform, not only is the chloroform coloured with the indole-red, but the supernatant fluid also remains red. These two red-coloured layers may now be separated and the upper stratum repeatedly washed with chloroform until no more colour can be extracted, *i.e.* until the chloroform remains colourless, whilst the upper stratum retains its red colour unchanged.

Group No. 2. (Purple or blue colour with a positive indole reaction.)

The reaction is similar to the above in its initial stages, but upon extraction with chloroform the indole-red is dissolved out leaving a purple upper stratum. This layer may now be separated and thoroughly washed with chloroform, the result being that when the last traces of indole-red are extracted the upper stratum is purple, with a more or less pronounced bluish tint, while the chloroform is as before perfectly colourless. The occurrence of this reaction is comparatively frequent.

If a series of tubes of peptone-salt solution be inoculated with a positive strain incubated for given periods and then tested daily, it will be observed that there is an increase, not only of the concentration of indole-red from day to day, but, also, of the purple colour.

At the same time, the differences which depend on the extraction being made shortly or a considerable time after the addition of the reagents, may be demonstrated. For example, in the following experiment, a series of tubes were inoculated with a positive strain and

incubated for periods of 24 hours, 48 hours etc., up to six days. At the end of 24 hours' incubation a culture was submitted to the ordinary technique without chloroform extraction and then divided into two equal parts *A* and *B*. Tube *A* was extracted with chloroform at the expiration of 30 minutes, and tube *B* was left standing for 24 hours before extracting. This process was repeated with each of the six cultures, after the periods of incubation previously mentioned.

The following facts were observed.

1. The intensity of both the red and purple colour was increased from day to day, and perfectly visible even on the first day.

2. In tube *A* the indole-red showed invariably a greater intensity than in tube *B*, whilst the inverse condition was observed with regard to the purple colour.

In passing, it may be noted that this purple colour is capable of destruction by the aid of suitable reducing agents and of being re-formed by adequate oxidation, also, that it is not produced unless the reagents are added in relative excess of that required to produce the indole reaction.

Group No. 3. (Formation of a blue colour with negative indole reaction.)

In this reaction the final result is the formation of a distinct blue colour without the admixture of any, extractible or non-extractible, red. Primarily there may be a slight reddening, but this colouration soon disappears and gives place to a more or less intense greenish tint.

The initial red colour is not extractible by chloroform or amyl-alcohol and is consequently not due to the presence of indole. If the extraction by means of chloroform or amyl-alcohol is made when the change of colour to green has already taken place, a separation into two layers is observed as before, the chloroform or amyl-alcohol layer being colourless while the watery layer is blue of an intensity varying, not only with the period of incubation but also with the particular bacterial strain. Chloroform extraction gives the better result. The use of sodium nitrite solution in the place of potassium persulphate produces a similar reaction, but the final colour is a clear green instead of blue. Similar to the phenomena of purple colouration, a greater intensity of the reaction is obtained if the cultures are left for several hours prior to extraction, after the addition of the reagents. A number of different strains of *B. coli* have been isolated which give this reaction; it has also been found in certain strains of *B. enteritidis* Gaertner, *B. paratyphi* A, *B. lactis viscosus* and *B. prodigiosus*.

When the reaction is but slightly pronounced, a comparison should be made with a tube of sterile peptone-water of the same stock, after addition of the reagents and extraction in identically the same manner, as a slight change of colour may also be observed in this way.

I wish to take this opportunity of expressing my gratitude to Associate Professor Glynn for the kind interest with which he has followed my investigations.

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THE EFFECTS OF DOSAGE IN TYPHOID VACCINATION OF RABBITS.

By J. HENDERSON SMITH, M.B.,

Assistant Bacteriologist, Lister Institute,

AND RALPH ST JOHN BROOKS, M.B., D.P.H.,

British Medical Association Research Scholar.

(With 7 Curves.)

It has long been known that the injection of very minute quantities of a suitable antigen is sufficient to induce a well-marked response in the animal into which they are introduced. Friedberger, for example (1902) demonstrated the appearance of antibodies in rabbits after the intravenous injection of $\frac{1}{2000}$ th part of 1 milligram of cholera vaccine, and the data which have accumulated since the introduction of vaccine-therapy, as also the phenomena of serum-anaphylaxis in the guinea-pig, show clearly enough the remarkable sensitiveness of the animal body to at least certain classes of material directly introduced into its tissues. These minimal injections, however, do not produce the full antibody-response, of which the animal is capable. Friedberger in the paper quoted showed that a larger dose was followed by the appearance of a larger quantity of antibody, and the experiments of Ascher (1901) with typhoid vaccine indicate that, broadly speaking, the production of antibodies increases with the size of the dose. This is indeed generally recognised to be true when the doses in question are quite small (cf. Wright, 1909); but it was one of the objects of the work recorded in the present paper to ascertain whether in the case of typhoid vaccine it is true for any really considerable range of dosage. Even for small doses the necessity of injecting the different doses into different animals (Cole, 1904) has made the point somewhat troublesome to establish. Individual animals vary very greatly in their response to injections of the same size, whether single or repeated, and Stäubli (1904), working with typhoid vaccine in guinea-pigs, went so far as to deny that there is any relationship whatever

between the quantity of bacteria introduced and the resulting formation of agglutinin. It is evident that conclusions can be based only on the average results of a considerable number of animals. Further it would seem to be evident that, if it exists at all, a direct relationship can hold good only within a definitely limited range of dosage. There must be a limit to the increase in production; and reactions of this kind, as estimated by the activity of the serum of the animals treated, tend even after repeated injections to approach a limit, beyond which they cannot be driven by any dosage. We have accordingly investigated this question afresh with typhoid vaccine and rabbits, using a wide range of doses and for each dose employing animals in numbers sufficient to allow of reasonable conclusions. The sera of these animals were tested in various ways detailed below.

The strain used for the preparation of the vaccine was one which had been isolated nine months previously from the urine of a typhoid carrier, who is known on definite bacteriological evidence to have been excreting the organism since an attack of enteric fever three years before. At the time of isolation, 0.1 c.c. of a 48 hours broth culture killed a guinea-pig in 24 hours. During the interval it had been kept in agar at room temperature, and its virulence appears to have altered a little between the time of first isolation and the date at which our experiments began; but that it remained fairly constant during the course of our work is shown by the following small table. The inoculations were made intraperitoneally.

Time	Dose	No. of guinea-pigs	Result
Beginning of experiments	2000 mills.	3	All died, 30 hrs.
	1000	3	Do. do.
	500	3	Do. do.
	100	3	2 died 30 hrs., 1 survived.
	50	3	2 survived, 1 died 12 days.
End of experiments	600	2	Both died, 30 hrs.
	300	2	Do. do.
	150	2	1 died 30 hrs., 1 survived.

This strain, which we shall call strain *S*, was the only one used in our experiments, with the exception of the tests for bactericidal action (see later). It was agglutinated microscopically by a powerful anti-typhoid serum in a dilution of 1:10,000, was actively motile, and gave the standard carbohydrate and other reactions. It was maintained by repeated subcultures in agar at room temperature, and this stock was never incubated at 37°. For use subcultures were made from a room temperature stock culture of 7—14 days old, and incubated

16—20 hours at 37°. The growth was then taken off in 0.85% NaCl solution, thoroughly mixed in a mechanical shaker, and the number of organisms contained in 1 c.c. of the suspension counted by a method which has an error of usually 2—5%, and at most 11%. The emulsion was then diluted with salt solution to the desired concentration. Throughout the whole of this work the suspensions were counted in this manner before use; and we consider that the increased accuracy obtained by working with suspensions of standard strength justifies the not very great additional labour involved.

The vaccine, which was freshly made for each series of experiments, was prepared by heating a counted emulsion at 60° for half an hour, and then diluting so that the required dose was contained in 1.5 c.c. This amount was then injected subcutaneously over the abdomen of each of ten rabbits of approximately equal weight, from each of which a sample of blood had been previously withdrawn. At intervals after the inoculation blood was taken from the marginal ear-vein of each of the ten rabbits, the serum taken off after 24 hours, and equal parts of the ten sera mixed together to form one sample. This was done on successive days after the inoculation, as indicated in the tables, and a series of samples obtained, each of which represents the whole of the ten rabbits taken together.

By this method of pooling the sera we hoped largely to obliterate the differences in the individual sera, which are particularly marked in the case of rabbits and typhoid bacilli, and to obtain the representative average required. A further and important advantage of the method lies in the fact that only a small quantity of blood need be taken from any one animal at each bleeding. In order to carry out the examination of the sera in the manner we proposed, about 5 c.c. of serum was necessary. This represents approximately 10 c.c. of blood, and the withdrawal of so large a quantity every day or every other day for a fortnight from an animal of the size of a rabbit would seriously affect the production of antibodies. Using ten animals we required only 1 c.c. of blood from each, and it was hoped thereby to minimise the effect of the repeated blood-letting.

In the earlier part of the work, each sample was tested for its content of various antibodies on the same day as we obtained the sera of which it was composed; and this method was adhered to throughout in the testing of the opsonic power of the whole serum, when it was essential that the serum should be fresh. But in other cases where heated serum was used, we subsequently adopted the more convenient method of

heating the samples for 30 minutes at 56° , and then keeping them in the cold and testing all or a number of them together on the same day. We thus examined a number of samples for, say, bactericidal action and agglutination on one day, and on the next day for, say, precipitating and tropic action, and so on. The error due to the different ages of the samples we did not find to be significant within the limits of time used, if they were reheated at 56° on the day of testing.

Five groups of rabbits were examined, each consisting of ten animals. To the first group were given 20 million bacilli per animal, to the second 100 million, to the third 500 million, to the fourth 2500 million, and to the fifth 12,500 million. The dose of typhoid vaccine usually given as a first inoculation to an adult man is 500 million, or from 7—8 million per kilo., taking the average weight of a man as about 70 kilos. The average weight of the rabbits used was $2\frac{1}{2}$ kilos., so that the first group received 8 million per kilo. or approximately the same dose, weight for weight, as is given to a man, and the fifth group received over 600 times as much. The doses were in nearly all cases well borne by the animals. Only two out of the 50 died within 14 days of the injection, both being in the first group with the smallest inoculation. There was a considerable loss of weight after the injections, but this did not increase proportionately to the size of the dose—*e.g.* a week after the injection, the average loss of weight in the animals of the first group was 10%, in those of the fourth group 5%, in those of the fifth group 8%. It is perhaps worth noticing that the greatest loss of weight and the only deaths occurred in the group with the smallest dose; but this cannot be attributed to a progressive change in the strain, since this group was in fact treated third in order of time after two of the other series had been completed.

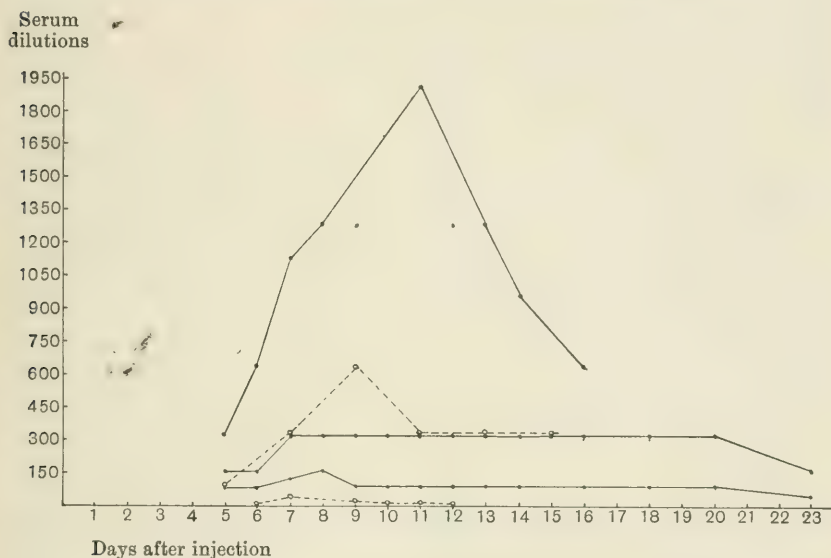
The samples of serum were examined for (1) their power to agglutinate the homologous bacillus; (2) their capacity to give a precipitate with an extract prepared from this bacillus; (3) their capacity to deviate complement, when conjoined with such an extract; (4) their bactericidal action; and (5) their capacity to promote phagocytosis. The results are tabulated under these five headings.

1. *Agglutination.*

This was observed macroscopically. To 1 c.c. of successive dilutions of the sample under examination was added 1 c.c. of a suspension of strain *S* containing 1500 million organisms per c.c. After thorough mixing the tubes were incubated two hours at 37° , when the results

were read; and after standing overnight at room temperature (about a further 18 hours) a second reading was taken. The latter was the reading adopted, but the earlier reading was sometimes of value in enabling one to decide definitely on slight differences between two samples. The standard chosen was the lowest dilution in which definite agglutination was visible.

In Table I is given an example of the complete examination of one group, and in Table II the results obtained with all the groups.



Curve 1. Agglutination. From above downwards the curves represent the series after 12,500, 2500, 500, 100 and 20 millions.

In the first two groups examined the serum was tested both fresh and after heating, but as identical results were obtained, the later series were tested with heated serum only. The results are perhaps better seen when shown graphically as in Curve 1. In most cases the dilution of the serum in the successive tubes of a test fell by one-half, so that a reading of, say, 1:40 means that the value of the serum lay between 1:40 and 1:80. The curves therefore cannot be smooth, and indicate with only approximate accuracy the change in agglutinin-content from day to day. They are of the familiar type, and, like the other curves representing our results, show the usual features of curves of antibody-production.

TABLE I.

Agglutination—Dose = 100 millions.

Serum-dilution	Before inoculation	Days after inoculation															
		5	6	7	8	9	10	11	12	13	14	16	18	20	22		
1:5	0	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
1:10	0	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
1:20	0	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
1:40	0	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
1:80	0	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
1:160	0	0	0	±	+	+	+	+	+	+	+	+	+	+	+	0	0
1:320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1:640	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

++ = complete, + = marked, ± = partial, 0 = no agglutination. Control without serum : 0.

TABLE II.

Agglutination: all series.

The numbers indicate the lowest active dilution of serum.

Dose of vaccine	Before injection	Days after inoculation															
		3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
20 × 10 ⁶	<5	<5	<5	<5	5	40	—	20	10	10	5	5	<5	—	—	—	—
100 × 10 ⁶	<5	—	—	80	80	120	160	80	80	80	80	80	80	—	80	—	80
500 × 10 ⁶	<5	—	—	160	160	320	320	320	320	320	320	320	320	—	320	—	320
2500 × 10 ⁶	<5	—	—	80	—	320	—	640	—	320	—	320	—	320	—	—	—
12,500 × 10 ⁶	<5	—	—	320	640	1120	1280	1280	—	1920	1280	1280	960	—	640	—	—

The conclusions which may be drawn from these results will be discussed later, but we may note that, as the dose of vaccine is increased, the maximum agglutinating power of the serum also increases, while the date at which this maximum is reached gets later.

2. *Precipitation.*

An extract was prepared from strain *S* as follows. Several Roux bottles of agar were inoculated, each with 1 c.c. of an 18 hours broth culture and incubated 48 hours at 37°. To each bottle was then added 20 c.c. of distilled water, into which the growth was swept off and the whole mixed. After heating one hour at 60° and allowing to cool, carbolic acid to 0.5% was added, and the suspension then frozen hard. It was slowly thawed at room temperature and shaken over night in a mechanical shaker at 37°. It was again frozen, thawed and shaken, and finally centrifuged. The supernatant fluid was distinctly opalescent but microscopically free from bacilli and was sterile. This stock was kept in the cold room, and one in ten dilution in 0.85% NaCl solution used for testing the samples of serum. To 1 c.c. of successive dilutions of the serum was added 1 c.c. of the extract-dilution, the whole mixed, and placed at 37° for four hours. A preliminary reading was then taken and the tubes examined again after standing all night at room temperature for final reading.

As in the case of agglutination the standard selected was the lowest dilution of the serum in which a definite precipitate was visible to the naked eye. This was usually easy to determine. The precipitum had the form of a slight loose delicate coagulum, of the appearance of a tuft of fine wool, never very copious but quite definite in suitable light and characteristic, floating up readily on gently shaking the tube. The results obtained are grouped together in Table III. (An example of a whole series is not given, as we obtained no irregularities or zone phenomena.)

TABLE III.

"Precipitation."

Dose, millions	Before injection	Days after injection																		
		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20			
20	0	0	0	20	—	10	10	10	10	10	5	—	—	—	—	—	—			
100	0	10	40	160	160	80	40	20	20	20	10	—	10	—	10	—	10			
500	0	40	320	320	320	320	240	240	240	160	160	—	80	—	80	—	80			
2500	0	10	—	40	—	80	—	80	—	40	—	40	—	20	—	—	—			
12500	0	160	160	160	1280	640	—	640	640	640	640	—	640	—	—	—	—			

Here again we notice a tendency for the maximal response to be reached at a later date with the larger inoculations, up to the dose of 2500 millions. With the highest dose, however, the maximum has regressed. Further the height of the maximum does not increase regularly with the increase in dosage. The injection of 2500 millions is followed by a serum-value of only 1 in 80, instead of the 1 in 600 or 700 one might expect. In any consideration of the significance of such variations in the regularity of response, it is necessary to remember that in the case of precipitation as of complement-fixation the results obtained are subject to an error which does not equally affect the other reactions examined. This lies in the difficulty of obtaining a constant antigen. A bacterial extract, kept in the cold and dark, undergoes a gradual alteration of the particles it contains, as is shown by the deposit which falls out as time goes on, and this change in the state of the particles affects the result of the interaction of the serum with the extract. When an experiment lasts over several weeks, as did those described, it is impossible to use the same extract throughout and still obtain results that are really comparable. It is necessary to make fresh extracts from time to time. But this proceeding introduces a fresh error, for no two extracts, though prepared in apparently quite the same way, are exactly alike in their reactions with the same serum. In our series the first and fourth were tested with the same extract, the second and third with a second extract, and the fifth with a third, all these extracts being prepared in the same way but at different times. Not much stress, therefore, can be laid upon the actual figures obtained, as indicating the relative strengths of the sera in question.

But however inexact these figures represent a relationship between the dose injected and the consequent production of "precipitin," they are at least approximately accurate for any one series and the extract with which that series was tested, and in this respect they are somewhat remarkable. It is the current view, at least in this country (Chapman and Welsh, and cf. Dean, 1911, who gives a number of references), that in precipitin reactions the precipitum formed, the actual material which falls out of solution, is derived mainly from the serum of the treated animal and only to a small extent, if at all, from the antigen. In accordance with this it is the general experience that, while the antigen may be diluted to an enormous extent before the reaction ceases to form a visible precipitate, dilution of the anti-serum is practicable only within a comparatively small range, even with high-grade sera. Thus it is a good serum which reacts in a dilution of 1:100, and sera

which react in dilutions of more than 1:300 are quite exceptional. But in our experiments, when the sera would ordinarily be considered as of low grade, we obtained precipitation in dilutions of 1:160, 1:320 and even 1:1280.

Again, while a serum of so high a value as 1:1000 would in the ordinary way permit of a dilution of the antigen to exceedingly high figures (1:100,000 or figures of that order), the reaction, which we obtained, ceased to appear when the antigen was diluted to 1 in 80. The whole of the samples of our last series were tested with extract-dilutions of 1:60 and 1:80, as well as 1:10, and no reaction was obtained with any sample in the 1:80 series, and only in a few tubes of the 1:60 series. Further, the nature of the coagulum, which has already been described, differs both from the copious flocculent precipitate and the general opalescence or turbidity obtained in strong and feeble reactions of the ordinary type. (Such precipitate we never obtained with any sample in a dilution of 1 in 2.)

These facts suggest that we are dealing here with a phenomenon which is quite distinct from the precipitation commonly described. They suggest that possibly the precipitate or deposit which we obtained is really an agglutination-phenomenon, an agglutination, however, not of bacilli but of the shells or particles of bacilli contained in the extract, and is not a precipitum in the sense in which that term is ordinarily used in connection with precipitinogen-precipitin reactions. This question requires a separate investigation, which is at present being undertaken.

3. *Complement-Fixation.*

We obtained results here with only one series, viz. after the injection of 500 millions. Two other series were also tested, those of the 20 million and 100 million injections, but no fixation whatever was obtained with any of the samples in these series by the technique adopted. Since we should have had only three determinations, even if (as was probable) the two largest doses had been followed by appreciable responses, and these would in any case have been subject to the limitations involved in the use of extracts, we did not examine the last two series, and include the results of the 500 million series only as an example of the kind of curve to be obtained. It is, however, not without interest that the small doses did not give us any reaction.

The technique adopted was essentially that in common use. To 1 c.c. of a 1 in 40 dilution of extract (prepared as described above)

was added 1 c.c. of successive dilution of serum, and 0.5 c.c. of fresh guinea-pig serum diluted 1 in 10. After 1 hour at 37°, 0.5 c.c. of a 5% suspension of washed ox blood-corpuscles was added, with 0.5 c.c. of a dilution of haemolytic amboceptor standardised with the same complement to contain 3 lytic doses. The mixture was then incubated two hours at 37° and, a preliminary reading having been taken, was left overnight in the cold, and then the final examination made. The haemolytic amboceptor was derived from rabbits immunised to washed ox-corpuscles, and the standard taken was the lowest dilution of the sample, with which definite inhibition of haemolysis occurred. The usual controls were always made.

TABLE IV.

Dose of vaccine	Before injection	Days after injection													
		5	6	7	8	9	10	11	12	13	14	15	17	19	
20×10^6	0	No reaction on any day.													
100×10^6	0														
500×10^6	0	10	20	20	20	20	40	40	20	20	20	10	—	0	

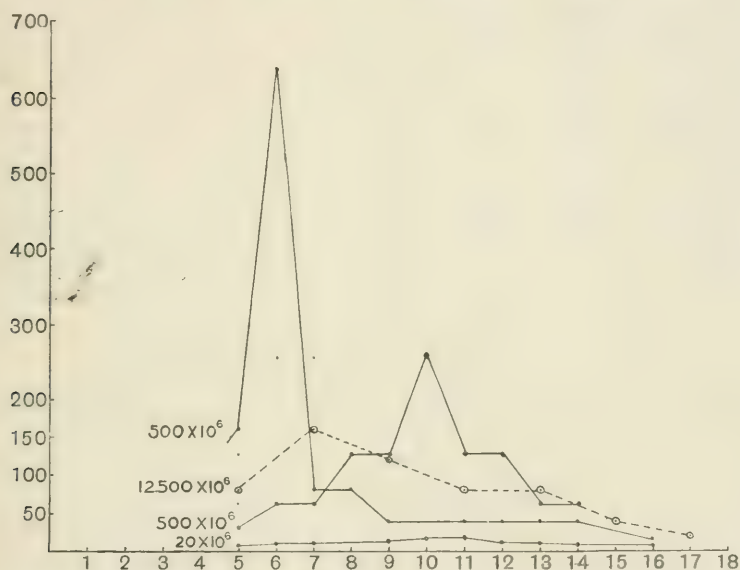
4. *Bactericidal Action.*

In testing this power of the serum we determined the smallest quantity of each heated sample, which in conjunction with a fixed quantity of fresh normal serum as complement killed all or all but one of a definite number of typhoid bacilli in a given time. Of each sample a series of dilutions in 0.5 c.c. volume was prepared, these dilutions being made in broth. To every tube was added 0.5 c.c. of a 1 in 2 dilution in salt solution of fresh normal rabbit-serum; and finally 3000 typhoid bacilli were added, contained in 0.5 c.c. of broth. The total volume thus amounted to 1.5 c.c. The necessary controls, which will be found detailed in Table V, were invariably prepared. We used as test-organism another laboratory strain of typhoid, and not strain *S*, which is rather resistant.

Immediately after mixing, two agar plates were made from the tube which contained only bacilli and the fresh normal serum, and also two plates from some one other tube. After incubating for 4 hours at 37°, two plates were made from every tube; the tubes were then filled up with broth and incubated overnight at 37°. In plating 5 drops were delivered into each plate from dropping pipettes standardised to deliver 0.02 c.c. per drop, only two pipettes being used for the whole

series (sterilised between each using). The plates were incubated at 37° , and the colonies counted, the mean of the numbers on the two plates being taken.

This technique differs from that usually employed in two points, the small number of organisms used and the large quantity of broth. The 1.5 c.c. of the mixture contained 3000 bacilli at the outset, so that on plating 5 drops or 0.1 c.c. 200 organisms were added to a plate. Of these 200 only 60–70% grew on agar at 37° , and the plates made at once after mixing show the easily counted number of 120–140 colonies. In a series of experiments carried out over several weeks,



Curve 2. Bactericidal action. (The scale for the 12,500 and 2500 million series is reduced one-fourth.)

in which the suspension of organisms was made sometimes by one of us, sometimes by the other, the actual counts nearly always lay between 100 and 140 in plates made at the beginning of the experiment, and the mean count in all experiments was 125. With this small number of organisms we found it impossible to use the small quantities of broth (*e.g.* 2 or 3 drops in a total of several c.c. of salt solution) which other workers have found sufficient. The bacilli died out steadily in the salt solution alone without the addition of any serum, and it was only when a considerable quantity of broth was present that their

numbers remained undiminished. Whether this is to be attributed only to an increased rate of multiplication induced by the additional broth or to an actual diminution in the death-rate was not determined.

In Table V are given the results of the examination of one series, and in Table VI a summary of all the series taken together. The curves are shown in Curve 2.

TABLE V.

Bactericidal action; injection = 2500 × 10⁶ bacilli.

Serum dilutions	Before injection	Days after injection					
		5	7	9	11	13	15
1 : 5	many	0	0	0	0	0	0
1 : 10	many	0	0	0	0	0	0
1 : 20	many	0	0	0	0	0	0
1 : 40	many	0	0	0	0	0	0.5
1 : 80	—	0.5	0	0	0	0	10
1 : 160	—	4	0	3.5	36	86	171
1 : 320	—	60	20	30	271	many	many
1 : 640	—	277	178	175	many	many	many
1 : 1280	—	—	many	many	many	many	many
1 : 2560	—	—	many	many	many	many	many

Fresh Normal Serum + Broth : after mixing, 128.

after 4 hours, many (about 320).

Fresh Normal Serum + Sample before injection (1 : 5) : after mixing, 135.

0.5 c.c. of all samples 1 : 5, and of fresh normal serum 1 : 2, in 5 c.c. broth : all sterile.

TABLE VI.

Bactericidal action: all series.

Dose of Vaccine	Before injection	Days after injection															
		5	6	7	8	9	10	11	12	13	14	15	16	17	18		
20 × 10 ⁶	not in 1	8	10	10	—	12	16	16	12	10	8	—	8	—	—		
100 × 10 ⁶	<2	64	128	256	128	128	256	128	128	128	64	—	64	—	<16		
500 × 10 ⁶	<2	128	256	256	512	512	1024	512	512	256	256	—	—	—	—		
2500 × 10 ⁶	<5	80	—	160	—	120	—	80	—	80	—	40	—	20	—		
12,500 × 10 ⁶	<10	640	2560	320	320	160	—	160	160	160	160	—	80	—	—		

From these figures it appears that here again we have an increasing maximum with an increase of dose. The fourth series, when the dose is 2500 millions, is distinctly out of sequence, and this point will be referred to subsequently, but the other series show a rise from 1 in 16 with the smallest dose to 1 in 2560 with the largest dose. As in the case of the precipitation-values, however, the actual figures can be taken only as indicating approximately the relative strengths of the

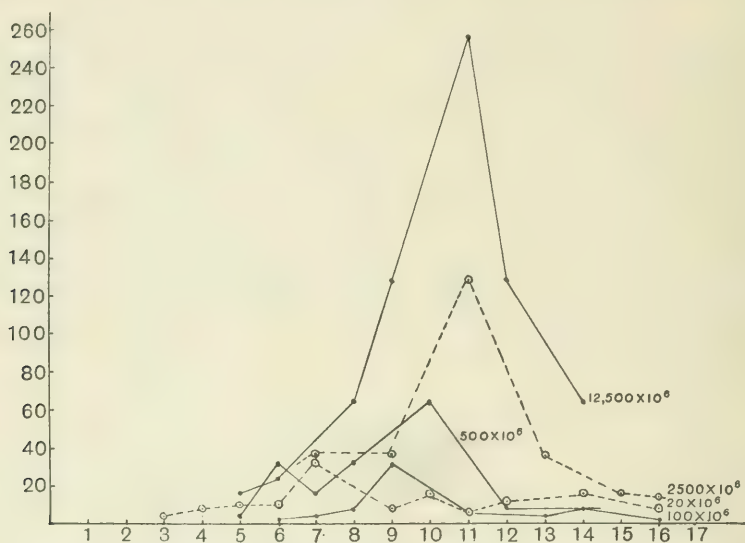
different series. These were necessarily tested with complement derived from different animals, and rabbit sera vary very markedly in their bactericidal power. Even when the pooled serum of two or three animals is used as complement (which was our usual practice), the difference in complementing power between two batches is still very marked, and a sample of immune serum will give a much higher value with one than with another. This is apparent in the controls of the normal serum alone. With the technique adopted, 0.5 c.c. of a 1 in 2 dilution of normal serum had considerable effect on the number of living bacteria found at the end of four hours, as compared with the numbers in a tube containing no serum at all. In most cases the numbers had only doubled or trebled themselves. But not infrequently the numbers actually fell, and on rare occasions all the organisms were killed. Experiments, in which diminution of this kind occurred, were rejected, and we accepted only those in which the bacteria in the complement-control tube had increased to two or three times their original number. But this variability of the power of the complementing serum makes it impossible to look upon the figures obtained as giving more than a general indication of the relative strengths of the maxima in different series.

5. *Phagocytosis.*

In testing the capacity of the samples to promote phagocytosis the dilution-method was employed (Klien, 1907). Successive dilutions of the sample were made and these dilutions were mixed with bacteria and leucocytes for 10 minutes at 37°. Smears of the mixture were then made, stained with Giemsa's solution and the average number of organisms taken up per leucocyte ascertained by counting in the usual way. From 100-300 leucocytes were counted of each mixture examined, the average number per slide being 120 in 80,000 cells. A control was prepared at the same time in which normal serum diluted 1 in 2 with salt solution replaced the sample-dilution; and the lowest dilution of the sample which gave a higher count than the control was taken as the value of that sample for our purpose. Human leucocytes were used, prepared in the way customary in the opsonic technique, and a bacterial suspension of 2000 millions in 1 c.c. (strain S).

Three methods were adopted in testing the activity of the samples. In the first the serum was used fresh after 24 hours on the clot, and

equal volumes of serum-dilution, bacteria, leucocytes and salt solution were mixed together, the control containing fresh normal serum. In the second, heated serum replaced the fresh serum of the first method, the control containing heated normal serum. In the third, heated serum was again used but complement was supplied by replacing the salt solution volume by one volume of fresh normal rabbit serum, the control containing heated normal serum instead of the immune-serum of the sample. The fresh normal serum used in the controls in the



Curve 3. Fresh serum phagocytosis. (The 12,500 and 2500 million curves are reduced one-half.)

first and third methods was derived from the same rabbit throughout, and the heated normal serum of the controls was the pooled serum of the rabbits of the same series before injection with the vaccine. As may be seen from Table VIII subjoined, normal serum induced remarkably little phagocytosis of strain *S*, indeed not much more than what occurred in salt solution alone, and the presence of complement made little appreciable difference.

As illustrations of the results obtained in this way we reproduce in Table VII the data of the examination of one series with fresh serum; and in Table VIII the figures obtained by all three methods with one sample. In Table IX are summarised the results of the examination of all the sera, the figures in the columns under each day indicating

the lowest dilution which gave more phagocytosis than the corresponding control. Cf. Curve 3.

It is of course a familiar observation that short exposure to moderate heat greatly reduces the opsonising power of an immune serum, without in general completely destroying it. This phenomenon is brought out in these figures got by the dilution method at least as clearly as by the usual method of comparing the phagocytic indices of undiluted serum in the fresh and the inactivated states. They further bring out, and even more clearly than is possible by the usual methods, the very marked influence of the addition of fresh normal serum or complement. This is best shown by an example, and we take the figures from the 500 million series on the 10th day after injection. Here the

TABLE VII.

Phagocytosis with Fresh Serum; Injection, 500 millions.

Serum dilution	Days after injection						
	5	6	7	8	10	12	14
1/1	5.26	—	—	—	—	—	—
1/2	2.68	14.41	7.63	—	—	2.74	—
1/4	1.27	—	5.60	11.28	—	2.55	2.70
1/8	0.72	3.20	—	—	11.84	0.91	1.64
1/16	0.32	1.52	1.47	1.83	—	0.51	0.31
1/32	—	1.29	0.63	1.08	2.66	0.42	—
1/64	0.35	0.88	—	0.67	1.51	—	0.13
1/128	—	—	—	—	0.56	—	—

Control of Normal Serum 1/2 taken as 1.00.

TABLE VIII.

Phagocytosis: 12th day after injection of 100 millions.

Serum dilutions	Fresh serum	Heated serum	Heated serum + Complement
1/1	6.73	0.64	—
1/2	2.65	0.49	—
1/4	1.15	0.25	—
1/8	0.26	0.23	—
1/16	—	0.10	2.84
1/32	—	—	1.34
1/64	—	—	0.84
1/128	—	—	0.42
1/256	—	—	0.35
1/512	—	—	0.20
Control	0.37	0.31	0.30

TABLE IX.

Phagocytosis by the three methods with all doses. The numbers indicate the lowest serum-dilution found.

Dose	Method	Days after injection																
		3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
20 millions	Fresh	4	8	10	10	32	—	8	16	9	12	—	16	—	8	—	—	
	Heated	—	No reaction obtained on any day.															
	Complemented	—	256	—	512	512	—	256	256	128	256	32	16	—	2	—	—	
100 millions	Fresh	—	—	1	2	4	8	32	—	6	6	4	8	—	2	—	—	
	Heated	—	—	2	—	4	4	8	8	2	2	2	—	—	2	—	—	
	Complemented	—	—	32	128	128	512	1024	512	—	256	128	256	—	—	—	32	
500 millions	Fresh	—	—	4	32	16	32	—	64	—	8	—	8	—	—	—	—	
	Heated	—	—	16	16	—	16	—	32	—	32	—	16	—	—	—	—	
	Complemented	—	—	128	2048	1024	1024	1024	2048	1024	—	—	256	—	—	—	—	
2500 millions	Fresh	—	—	—	—	64	—	64	—	256	—	64	—	32	—	28	—	
	Heated	—	—	—	—	16	—	64	—	64	—	32	—	16	—	—	—	
	Complemented	—	—	—	—	512	—	1024	—	4096	—	512	—	256	—	64	—	
12,500 millions	Fresh	—	—	32	48	—	128	256	—	512	256	—	128	—	—	—		

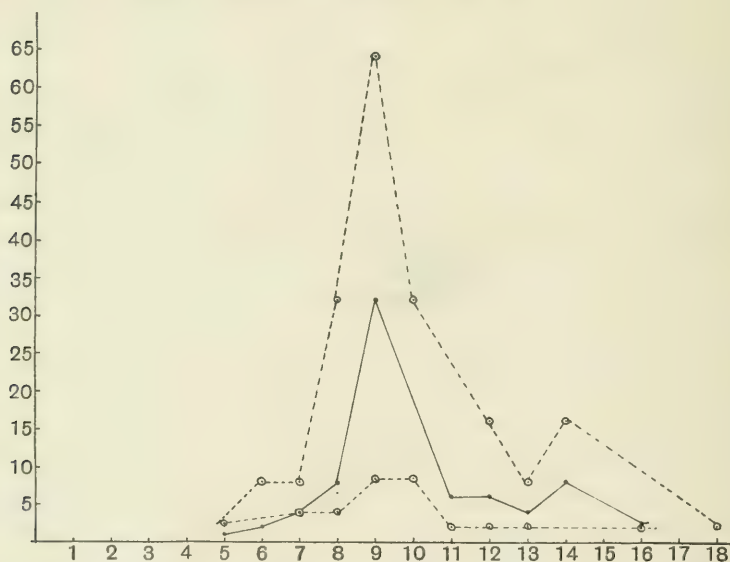
heated sample ceased to be more active than heated normal serum, when it was diluted beyond 1 in 32. On the addition of fresh serum, however, the heated serum of the sample proved much the more active of the two, and it was not until the sample had been further diluted down to 1 in 2000 (*i.e.* 64 times more) that its action again equalled that of the normal heated serum. There is here no question of addition or summation of the effect of the heated serum acting alone upon the effect of the fresh serum also acting alone, since the actions of the two heated sera, the normal and immune, are equal at 1 in 32, and the fresh serum should affect both equally if it were a mere summation effect. There can be little doubt that the power of the heated immune serum is greatly and (as one knows on other grounds) specifically reinforced by the addition of the fresh normal serum or complement. The sensitisation of the bacteria, then, in the experiments where fresh serum is added, is a process of the complement-amboceptor type¹, and involves at least two factors, one thermolabile contained in the fresh serum and the other thermostable in the heated serum.

We should expect then the reaction of the fresh unheated immune serum to be also of this type, and our figures supply a further confirmation of this view, which is that of G. Dean (1907), Cowie and Chapin (1907), and other workers. In other complement-amboceptor reactions, it is known that it is the so-called amboceptor which rises and falls during the process of immunisation, the complement remaining relatively little affected. If that is true here, the variations in phagocytic power of the serum from day to day are due to variations in the thermostable factor, and in our two methods of testing with whole fresh serum and heated serum to which complement is added, we are really measuring the same thing, *viz.* this thermostable factor. The curves showing the variation should therefore be similar for the two methods and follow the same course, rising and falling together throughout. We can hardly expect them to be exactly alike, since, apart from the considerable experimental error, dilution by the one method reduces both the active factors, by the other only the thermostable factor, but there should be a general parallelism of rise and fall. This is what in fact we do find. In Curve 4 the figures for one series are plotted in the same chart, and it will be seen that the similarity between the upper two curves is very marked. The other series show the same general agreement

¹ We use these terms only as the names currently employed to describe phenomena of a certain class; and not as terms which in any way represent the mechanism of the reactions concerned.

TABLE X.

Dose	Agglutina- tion	Precipita- tion	Bactericidal action	Phagocytosis		
				Fresh serum	Comple- mented	Heated serum
20×10^6	40	20	16	32	512	—
	7th day	7th day	10-11th day	7th day	6-7th day	—
100×10^6	160	160	256	32	1024	8
	8th day	7-8th day	7 & 10th day	9th day	9th day	9-10th day
500×10^6	320	320	1024	64	2048	32
	7th day <i>et seq.</i>	7-9th day	10th day	10th day	6 & 10th day	10-12th day
2500×10^6	640	80	160	256	4096	64
	9th day	9-11th day	7th day	11th day	11th day	9-11th day
$12,500 \times 10^6$	1920	1280	2560	512	—	—
	11th day	8th day	6th day	11th day	—	—



Curve 4. Phagocytosis: by all three methods. The curves from below upwards at their highest points are in order (1) heated serum; (2) fresh serum; (3) complemented serum (reduced to one-sixteenth). Dose: 100 millions.

between the curves of the fresh serum and the complemented serum; and we believe that they represent the same phenomenon measured in different ways, viz. the variation in the thermostable factor.

In the curves of this kind where the results of all three methods of testing are plotted on one chart, it is noticeable that while the two

upper curves follow the same course fairly closely, the third curve, that of the heated serum alone, does not show nearly so good an agreement. Its maximum does not coincide exactly with theirs in time, and it does not follow their irregularities very closely. The figures are perhaps not very good (the actual counts of the bacteria in the leucocytes are always low and therefore subject to a considerable error), and the differences are not so striking that we should care to lay very much stress on them. If however they are regarded as accurately representing the state of the sample, they have very considerable significance. We have seen that the capacity of an immune-serum to promote phagocytosis is mainly due to a complex action of the complement-amboceptor type. But when this is admitted, it is no longer necessary to suppose that the phagocytic power of heated serum is due to the amboceptor of the complement action. It may equally conceivably be due to the action of quite a different substance acting alone and always independently of complement, although its presence is masked by the more powerful complement-amboceptor action, if the latter is available. This is the view of Neufeld, who gave the name of bacteriotropin to this independent, thermostable body, and it agrees with the common experience in other reactions that amboceptor is typically ineffective by itself. On this view there would be no reason to expect an agreement between the curve of heated serum and the other two curves, and the lack of coincidence which we actually find is at least suggestive. The differences we have met with, however, are not sufficiently striking to permit of any very definite conclusions from these experiments.

The results of the separate investigations of these various antibody-reactions are brought together in abbreviated form in the last table, No. X. In this the maximum value of the pooled serum of the 10 rabbits is tabulated opposite the dose of vaccine to which it corresponds, and then is also tabulated the date after injection on which that maximum value was obtained. From these figures some conclusions may be drawn, which for the sake of clearness are grouped together under three headings, (1) the date of the maximum response, (2) the relation of the response to the dose, (3) the relation of the antibodies to one another.

1. *The date of the maximum response.* It is apparent that in the agglutination, phagocytosis and "precipitation" series, the maximum value of the serum after injection is reached at a date which gets progressively later, as the dose is increased. The date for the last precipitin value has regressed but this is the only exception in the

series. A similar postponement of the apex of the opsonic curve has been already described by Wright as occurring in man after too large a dose of bacterial vaccine, but so far as we are aware has not hitherto been noticed in connection with agglutination. Wright connects this delay with the occurrence of a negative phase which follows shortly after the injection. During this phase the serum-value is depressed below that which it possessed before the injection was given and this condition persists for a time, which varies, generally speaking, according to the magnitude of the overdose. When this period is over the serum-value rises in the typical case beyond its original value and reaches a maximum in the usual way; but this interval of depression naturally makes itself felt by a delay in the arrival of the maximal value. The phenomenon is most easily demonstrated in individuals, whose serum already possesses a fairly high value and was shown by Salomonsen and Madsen, so long ago as 1897, to occur in antitoxin horses after the injection of diphtheria toxin. In our animals we did not examine the serum before the 3rd or in most cases the 5th day after injection, by which time the negative phase, if present, might well have passed off, and it would in any case have been rather difficult to demonstrate in animals previously normal with serum-values so low as those we found before injection. The curves, however, do not suggest that the four days' delay in reaching the maximum value with the largest dose is due simply to a negative phase of corresponding duration. If that were so, we should expect the curves with late maxima to cross the base line later than the curves with early maxima, and therefore to intersect these early curves during the rise to the apex: and this does not appear to be the case, *vide* the curves of fresh serum phagocytosis, no. 3, and agglutination, no. 1. We incline rather to attribute the delay to a longer period of absorption of the large dose from the site of injection. The small dose is absorbed comparatively rapidly from the subcutaneous tissues; whereas with a large dose this process takes a longer time. There is therefore a more prolonged stimulation of the cells in the latter case, with a correspondingly prolonged period of rise in the curve. This view agrees better with the fact that the curves do not intersect, but as a general rule the curve with the higher maximum is higher throughout on each of the observed days than the curve with low maximum—at any rate during the period of rise. The phenomenon recalls the delay in the development of the anaphylactic state after a large dose of sensitising antigen.

No such delay occurs with the bactericidal action. On the contrary

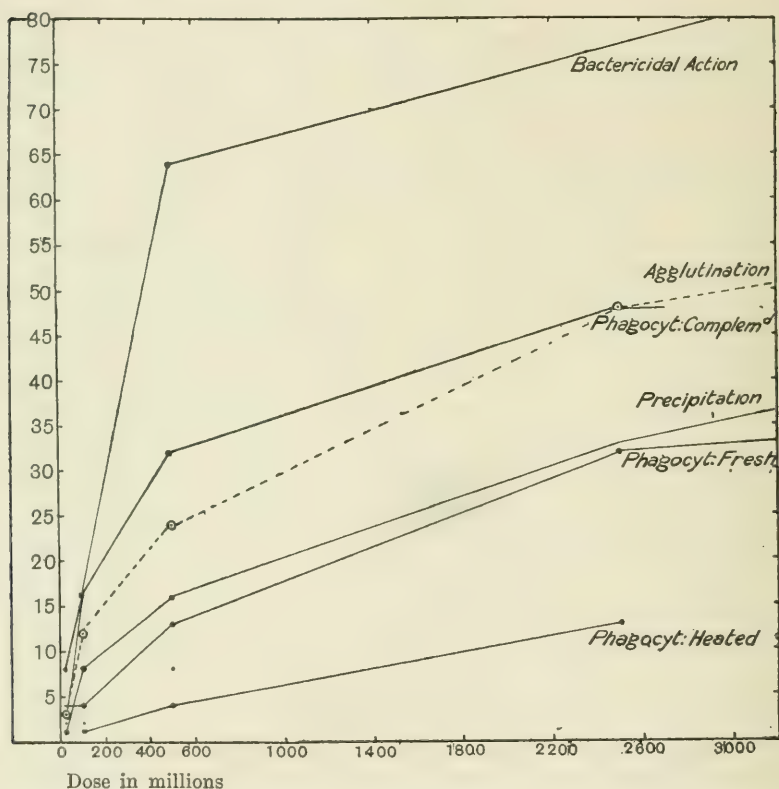
the maximum is reached sooner with the larger doses than with the smaller, and sooner with the largest than with any of the others. We shall return to this point immediately.

2. *The relation of the response to the dose.* From the figures in the table it will be seen that the maximal response in general increases with increasing dose. In the cases of agglutination and phagocytosis this increase proceeds uninterruptedly and apparently fairly regularly from the lowest dose to the highest, but in the columns for "precipitation" and bactericidal action the sequence is broken by the results of the 2500 million injections. From what has been already said the fall of the precipitin value with this dose may be due simply to a change in the extract with which the samples of this series were tested, and the fact that it is in this same series that the fall of bactericidal power occurs, though curious, may be simply coincidence. The low value of the bactericidal action, however, is not due to an error of this kind. We repeated the examination of the samples of this series more than once, and, although for reasons already discussed the maximal value found varied a little, it never reached a figure so high as that of the 500 million series, and was always found to occur in the same sample of the series. In these tests we met with nothing of the nature of a zone phenomenon nor any indication of a Neisser-Wechsberg reaction (which we obtained in none of the series, although attention was specially directed to this point); and the smoothness of the curve (see Curve 2) is against the probability of such an explanation. Any lowering of the curve due to excess of amboceptor should disappear as the amboceptor falls again after passing the apex, and the curve would show a double peak instead of the steady fall it actually exhibits. There seems no reason to doubt that the figures given represent with fair accuracy the actual value of this series relatively to the others, if the method of testing is valid at all, and that a less reaction followed this injection than followed a higher or lower dose.

The explanation of this irregularity is not obvious. We had anticipated that such a fall in the serum-values might be obtained with high doses of vaccine, if we reached that limit to the response which, as we saw on p. 77, there is reason to expect. The result of the highest dose, however, shows that this limit had not been reached, and the large difference between the two top doses (2500 and 12,500 millions) makes it improbable that the low reaction is a suggestion that we were already approaching that limit with the lower dose. It is possible that in this batch of 10 rabbits chance had brought together an unusual

proportion of animals with low reactive power, though the results of the agglutination and phagocytosis experiments do not support this view. Whether it is really a chance result or has some actual significance could be satisfactorily settled only by repetition of the experiments.

In Curve 5 the results are plotted on a diagram which brings out the relationship between dose and effect more clearly than the table. (The irregular values in the "precipitin" and bactericidal series are

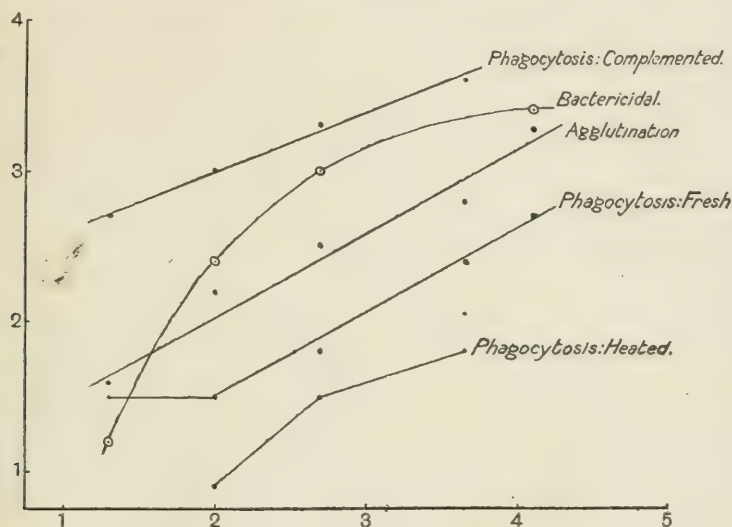


Curve 5. The maximum response is plotted against the dose. The scale is reduced and altered for the different antibodies to bring them all within one diagram.

omitted.) It appears from this that while the effect is still increasing with the increase of dose, relatively it gets less and less with each increase. The course of the individual curves clearly suggests that if we extended our range of dosage considerably but not indefinitely higher, we should reach a point beyond which a further increase of

dose would produce no further increase in response, *i.e.* we should reach the anticipated limit.

In Curve 6 these same results are plotted in a different way, which illustrates also a difference between the bactericidal and the other functions investigated. Here the logarithms of the serum-values are plotted against the logarithms of the doses. From the nature of our data, in which the value of the various samples was for the most part determined with only a rough approximation to the real value, we cannot expect to deduce with certainty any general mathematical expression correlating the dose with the effect, nor to find any very good agreement between calculated and observed values, if such an



Curve 6.

expression were found. It will be seen from this figure, however, that the curves of the various antibodies (with exception of bactericidal action) tend to take the form of straight lines, and do so within the range of error of our method. If for each antibody we calculate the values according to the formula $\text{Effect} = K \times (\text{Dose})^n$, when by "effect" we mean the maximal effective dilution and K and n are constants, the value of K is really not far from constancy. For example, with $n = 0.43$ the values of K differ by less than 0.5 % for the complemented phagocytosis figures, and with $n = 0.55$ by 30 % for the agglutination figures. But if we take "effect" as representing the amount adsorbed and "dose" as representing original concentration of the antigen, this

is a form of the well-known adsorption-formula, and we have the relationship between dose and effect following the rules of adsorption phenomena.

This result is not so improbable as it might at first sight appear. The figures in our tables indicate the dilution in which the serum was effective. They accordingly express directly the strength of the serum, and this varies directly with the concentration of the antibody. Now it is reasonable to suppose that the greater the stimulus to antibody production (using stimulus in its widest sense) which the cells experience, the greater within limits will that production be. In measuring the strength of the serum, then, we are indirectly measuring the stimulus applied to the cells. But this stimulus is supplied by the antigen taken up by the cells, and we have therefore in the strength of the serum an expression of the amount of antigen taken up by the cells—*i.e.* the amount bound. This quantity itself depends on the quantity of antigen actually offered to the cells, *i.e.* upon the injected dose or original concentration, and it is not at all impossible that the taking up of antigen by the cells of the tissues from the body-fluids is an adsorption process. If so, then the relationship between the amount adsorbed and the original concentration will be as stated in the above scale, when the amount of adsorbing substance, *i.e.* the cells, is kept constant, as in our experiments it may be taken to be. Accordingly, since the measure of the antibody produced is an indirect measure of the amount of antigen bound, we might expect the relationship of $\text{Effect} = K \times (\text{Dose})^n$ to hold good. The process may be compared to a haemolytic experiment in a test tube, if we assume for the moment that the taking up of the lysin by the cells is an adsorption process. In this case we might measure the amount bound indirectly by the effect produced, *viz.* the haemolysis, instead of directly by determining the amount left free. In the case of antibody-production we measure in a quite similar way the amount bound by the effect produced, *viz.* the antibody formed, the animal body taking the place of the test tube and the cell of the tissues the place of the red corpuscles.

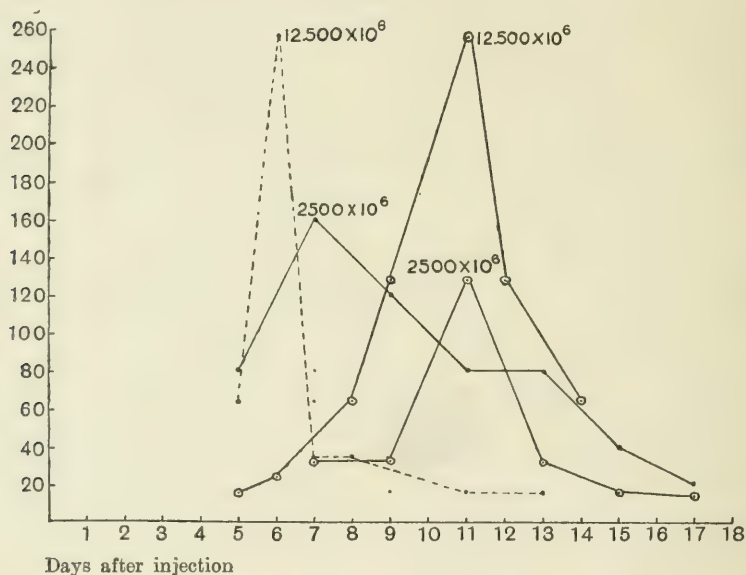
Our data then may be taken as in some degree suggesting that the process of fixing antigen by the tissue-cells is an adsorption process. They are unfortunately not sufficiently precise to do much more than suggest it: and the figures may admit of other interpretations. It would appear possible, however, to arrive in this way at some idea of the nature of certain processes occurring within the animal body, or at least of the rules which govern them.

3. *The relation of the antibodies to one another.* It has already been pointed out, when speaking of the date at which the maximum serum-value was reached, that in this respect the bactericidal action differs from the other antibodies investigated. Instead of the date getting later as the dose is increased, the bactericidal maximum comes earlier with increasing dose, and the difference is so decided and so consistent throughout the series that it appears impossible to attribute it to merely accidental circumstances. A similar distinction of the bactericidal function is met with in the relation which its maximum response bears to the dose. In Curve 6 it is evident that while the plotting of the other antibodies gives lines which do not vary greatly from straight lines, the plotting of the bactericidal values gives a line which is quite definitely curved. These differences point to a real distinction between the bactericidal function and the others, and without going into a detailed discussion of the subject something must be said of their bearing on the question of the identity of the various antibodies.

As soon as it was shown that after immunisation with a single antigen the serum of the treated animal had acquired not one only but two or more new properties, the question arose at once whether each property had a distinct substance corresponding to it or whether there was only one single substance which under different conditions acted in different ways. As all attempts have as yet failed to answer this question by direct means, such as isolation of different substances carrying the characteristic properties, the problem has been attacked indirectly, and our data supply an excellent example of the kind of evidence on which reliance is usually placed. In Curve 7 are charted the bactericidal and phagocytic values of all the samples after the injection of the two largest doses of vaccine. It will be seen that the curves of the two functions show no sort of agreement for either of these doses. In both cases the bactericidal action comes to a maximum some days before the phagocytic action and has fallen again to a relatively low figure while the other is still rising. (A similar disagreement occurs with the low doses, but in these it is the phagocytic action that is falling while the bactericidal is rising. With the middle doses the curves are not so dissimilar, the maximum in both cases falling on the same day; and this fact, while illustrating the unreliability of arguments based upon an apparent agreement, may explain the results of those who, like G. Dean (1907), have found the two functions to vary together.) If these curves represent the antibody-content of

the serum with anything approaching accuracy, it seems hardly possible to avoid the conclusion that the two functions are due to different substances. It is on evidence of this kind that the identity of two antibodies is denied, not only in the case of bactericidal and phagocytic action but for most of the known antibodies.

The validity of the evidence manifestly depends on the condition stated, that the points on the curves do represent with something like accuracy the antibody-content of the different samples of serum. They need not, however, represent this content absolutely. It has recently



Curve 7. The curves with maxima on the 6th and 7th days are those of bactericidal action; the curves with maxima on the 11th day are those of phagocytosis with fresh serum after the corresponding injections. (Scales reduced.)

been very clearly shown by H. R. Dean (*loc. cit.*) in the case of precipitin and complement-fixation reactions, that a single simple testing gives no accurate measure of the absolute capacity of a serum, and this fact is recognised by most persons familiar with the titration of haemolytic sera. Probably in no case up to now has the absolute content of a serum been accurately determined for any antibody, not even for diphtheria antitoxin. The effect produced is so dependent on the manner of obtaining it (cf. the Danysz phenomenon) that it may be made to vary in different circumstances within limits which are

quite undefined. It is sufficient, however, for the argument as to identity that the values of different sera shall be determined with fair accuracy relatively to one another.

Now in the case of the two functions shown on our chart there is an obvious difference in the method of obtaining the results, which may at first sight make comparison seem invalid. The bactericidal values are obtained upon a titration of the serum with the bacteria which lasted for four hours; in the phagocytic methods only 10 minutes were allowed for the process. In the former the action was nearly at an end, in the latter it was interrupted before it was nearly complete. It is not unusual in immunity for a reaction to run, at least for a time, more quickly with a certain dose of serum than it does with doses higher or lower. In a haemolytic series, for example, it may sometimes be seen that one or two tubes show more advanced lysis after, say, 15 minutes than the tubes above or below it, while the final results show no irregularity: and similar phenomena are known to occur with ferments. It is conceivable that in taking different times for our two reactions this may be misleading us here. We do not, however, think that this objection is really sound. The bactericidal action is practically over, and if an error of the kind indicated is taking place, it would lie in the short-time phagocytic series. Apart from the fact that the phenomenon has not, so far as we know, been observed in phagocytosis, to explain the different course of the curves on this ground would mean that the displacement of the maximum occurred regularly and progressively in each of the successive samples from the 6th day to the 11th, that it occurred similarly and regularly in all three forms of the phagocytosis tests in spite of their different character, and it would further involve a quite remarkable raising of the true maximum above the apparent one, already surprisingly high. Moreover the objection does not apply to the long-time agglutination tests, yet here the date of the maximum value in the different series agrees with the phagocytosis date and not with the bactericidal date.

A criticism of another kind may be brought forward on lines indicated by H. R. Dean (*loc. cit.*). Dean, working with precipitation and complement-fixation, showed that in these cases to every dose of antiserum there corresponds a dose of antigen with which a maximum reaction can be obtained. This dose of antigen is not inversely proportional to the dose of antiserum but may, at least within certain limits, fall as the antiserum falls. It follows from this that when equal volumes of two sera of different strengths are tested against a fixed

quantity of antigen, the resulting action in the two cases will depend on the quantity of antigen selected. This may chance to be chosen so that it is the optimal or nearly the optimal amount for the weaker serum, which therefore gives nearly its maximum reaction. It is in that case not the optimal amount for the stronger serum, and the maximum reaction possible for this serum is accordingly not obtained. The reaction got may in fact be actually less than that got with the weak serum, which therefore appears to be the stronger.

It also follows from the same fact that the method of titrating a constant and equal volume of the two sera against amounts of antigen which are reduced until a desired effect is produced (the method hitherto usual in precipitin experiments) can give no immediate measure of the relative values of the two sera. For as the result of such an experiment we obtain only two isolated facts which cannot be immediately compared with one another. We learn that with a fixed but unknown quantity of antibody contained in a measured volume of serum A a certain amount of antigen is required to produce a desired effect, and that with a fixed and also unknown but different amount of antibody in the same volume of serum B a different amount of antigen is required to obtain the same effect. But no conclusion can be drawn from the difference in the amounts of antigen required as to the relative strengths of the two sera: for neither the quantity of antigen nor the quantity of antibody is equal and constant in the two halves of the experiment, and there is no basis of comparison at all.

This objection loses its force when the more usual method is adopted of keeping the quantity of antigen constant and equal in both series, while the sera are progressively diluted. If with a fixed quantity of antigen there is one quantity of antibody, and one only, which gives a maximal reaction, it is necessarily true that in a series of successive dilutions of the two sera some one of these dilutions will in each series contain this optimal amount of antibody or nearly so (unless indeed one or both sera are so weak as not to contain it even undiluted, or unless the mere act of dilution in itself alters the condition of the antibody—a contingency not at all unlikely but which has still to be proved). With this optimal dilution the maximum reaction will be obtained in both series, and will be nearly the same in both series. But, since in order to arrive at this optimal amount less dilution of the weak serum will be necessary than of the strong, the maximum reaction will occur higher up in the series of the weak serum than in the series of the strong serum, which will therefore not only be but will be shown

to be the stronger. This will hold good for any dose of antigen. The larger this dose, the larger may become the necessary amount of antiserum, but the stronger serum will still appear the stronger. In the extreme case of a relatively very large dose of antigen, the weaker serum may be so weak as not to contain even when undiluted the requisite amount of antibody (in the volume chosen). We shall then not obtain a fair measure of the relative strengths of the two, but it will still be apparent which is the stronger.

It is not even necessary in practice to determine the dose of serum which in the two cases gives the actual maximum reaction possible. A selected reaction-result of less intensity is sufficient if care is taken to exclude a possible source of error. With a fixed quantity of antigen it may no longer be true to assume that there is one and only one quantity of antibody which will give the selected reaction, if this is less than the possible maximum. There may be two such quantities, one above and the other below the quantity of antibody required for maximal reaction. One must ensure that one does not determine for the one serum this supramaximal and for the other the submaximal quantity, and this is effected by sufficient extension of the serial dilutions below the maximal point.

The results of our testing may therefore be held to give an approximately accurate indication of the relative strengths of our serum-samples, even if it should eventually be proved that the relationship between antigen and antibody, which Dean has found for precipitating and complement-fixation reactions, holds good for other antibodies also. There seems to be at present no sufficient reason for doubting that a rise or fall in our curves does represent a rise or fall of the corresponding antibodies in the serum-samples, and that the bactericidal function should in the meantime be considered as due to the action of some other substance than the phagocytic function.

Although there is more or less agreement that a greater production of antibodies is obtained from a greater injection of antigen, we have found little in the literature beyond the opsonic evidence already referred to, which bears on the question of variation in the period at which the maximum is reached. Such evidence as there is hardly supplies suitable material for comparison with that brought forward in this paper, partly because most workers have used single animals and partly because they used living organisms measured in terms of broth-cultures or of standard-loops. From the series of papers by Leishman and his

colleagues, however, there can be extracted an indication of the date of the maximum reached after the injection of 20 millions of a typhoid vaccine. They used pooled rabbit sera (two or three animals) and accurately standardised doses. Unfortunately for our purpose, however, these small injections were always followed by a second larger dose and it is rarely possible to determine accurately from their charts when the maximum point after the first injection is reached. Further, it is not always apparent whether they were using broth-cultures or emulsions from agar: and the difference may make an important difference in the result. On the whole, however, it appears as if the summit of the curve after injection of 20 millions or 40 millions came later than in our experiments, about the 10th or 11th day, or perhaps later in the case of agglutination.

It is to be remembered in comparing results of such experiments that the strain used must make a distinct difference in the effect produced. This effect depends, *ceteris paribus*, on the quantity of antigen injected. But there is no reason to suppose that, say, 20 millions of one strain of typhoid bacilli contains the same amount of antigen as 20 millions of another strain, whether equally virulent in the living condition or not; and the accumulating evidence of other small differences in different strains of varying history suggests that this is unlikely to be the case. There is in fact a current belief that different strains of organisms vary considerably in antibody-producing power. The varying degree of resistance in different strains will also affect the readiness with which the antigen is dissolved out of the bacterial bodies. We should accordingly not expect with another strain of typhoid to obtain figures either for date or value, which were the same as those in the above tables; although it would seem probable that for any one strain the increase and the delay will be found to occur with increasing quantity of antigen.

SUMMARY.

Following the subcutaneous injection of typhoid vaccine into rabbits the average antibody response was estimated from day to day, and found to vary for agglutination, bactericidal action, phagocytosis (including tropins), "precipitation" and probably for complement-fixation according to the dose of vaccine.

As estimated by the maximal value reached by the serum the response increases with increasing dose in nearly all cases. This increase becomes relatively less and less as the dose increases, and its progress suggests

that with a sufficiently large dose a limiting-value might be reached, which if not actually a true limit would be practically one.

The observed relationship between dose and effect is such as to suggest that the combination of antigen and reacting-cell takes place in accordance with the rules governing adsorption processes; but this does not hold for the response in bactericidal power.

With increasing vaccine-dose the date at which the maximal value is reached gets later for the various antibodies, with again exception of the bactericidal power.

The precipitation observed differs from the usual precipitation reaction and is perhaps really an agglutination-phenomenon.

Phagocytosis was observed in three ways, and the bearing of the results on the mechanism of the action of the antibody concerned is considered; and the relationship between the bactericidal and other antibodies is also discussed.

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N^o 29

ON THE BACTERIOLOGY OF ASYLUM DYSENTERY.

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THIS report contains the results of an investigation of the stools and blood sera of acute and relapsing cases of dysenteric diarrhoea in a large asylum in the Midlands.

In England Shiga's bacillus was isolated by Eyre (1904), from cases in Claybury Asylum. He found six cases out of nine infected with *B. dysenteriae* Shiga, and in some of these cases bacilli of the Flexner type were also present though the patients' sera agglutinated the former to a greater degree than the latter. Altogether Eyre found the Flexner type in 21 out of 44 cases of asylum dysentery so that even in 1904 this type predominated. As far as I can ascertain *B. dysenteriae* Shiga has not since with certainty been found either in Claybury, though investigated by Candler and Dean (1910), or in any other English Asylum.

McWeeney's bacillus isolated from an Irish Asylum in 1905 belongs to the mannite type though differing from Flexner's bacillus in not producing indol. (Probably identical with *Bacillus* "Y.")

The bacilli isolated by Aveline, Boycott and Macdonald in 1908 from an Asylum near Taunton were all of the mannite type and it is worth noting here that they all gave slight to considerable amounts of indol and the majority did not ferment maltose till late.

Macalister (1910) investigated the same Asylum with which the present report deals and found numerous bacilli of the mannite type, some fermenting sorbite, others not.

Contemporaneous with this work on Asylum dysentery is the work of Morgan on Infantile Diarrhoea (1906-1909). In a small number of

cases of summer diarrhoea bacilli were found which resembled the mannite type but mainly on agglutination characters Morgan was dubious as to their admission to the Dysentery group. These bacilli fall into three sub-groups called by Morgan Nos. 3, 4 and 4a (see Table C). The non-motile mannite-fermenting bacilli which I have isolated from Asylum dysentery all belong to these three sub-groups and it seems highly probable that these are at least very closely related to those found in Infantile Diarrhoea, especially as Candler and Dean's strains from Claybury Asylum also correspond to Morgan's types No. 3 and No. 4a (see Table C).

Asylums being almost the last stronghold of Dysentery in this country (excepting occasional sporadic cases (Marshall 1909) often of imported origin, and occasional small house epidemics) and those strains isolated of recent years being probably descendants of those which formerly caused so much more widespread epidemics, it seems somewhat unreasonable to regard a bacillus as a true Dysentery bacillus or not, according to whether it corresponds with a bacillus found in another part of the world, separated possibly from common ancestors by many generations, subject to different climates and other factors which might lead to variations in character.

It has been shown that mannite-fermenting strains isolated from foreign sources differ considerably among themselves when thorough examination of their serological and biochemical properties is carried out (Morgan 1911), and in the case of British strains of the mannite-fermenting group a similar lack of fixity of character is revealed. Unlike *B. typhosus*, which is an organism characterised by great stability of character irrespective of its place of origin, the mannite-fermenting *B. dysenteriae* would still appear to be undergoing evolutionary changes.

As to the methods used in this investigation it is only necessary to state that specimens of faeces were obtained as a rule through the post and plated out on MacConkey's lactose agar. A number of pale colonies were picked off into mannite peptone water. Tubes giving acid and gas were thrown out; those giving acid or no change were further examined. By this method the Gaertner group and lactose-fermenters were excluded, whilst Dysentery and Morgan's bacilli Nos. 1 to 5 (see Table C) were found if present. The patients' sera were examined if possible at the same time and at a later date, as agglutination was often found to increase after the early acute stage. The results of this part of the work are summarized in the following table (Table A).

TABLE A.

Case Number	Character of Faeces	Agglutination of Bac. Y by patient's serum	Non-lactose fermenters not giving gas on Mannite
1	—	+1/50	<i>B. dysenteriae</i> . Mannite type.
2	Soft yellow stool, no blood	+1/100	Non-sorbito group
3	Dark green fluid, no blood	+1/40	" "
4	Yellow stool with blood and mucus	—	" "
5	Greenish yellow fluid, no blood	+1/100	" "
6	Only blood and mucus ...	—	" "
7	Only blood and mucus ...	+1/100	Sorbito group (also Morgan No. 1)
8	Brown liquid, no blood ...	—	"
9	Blood and mucus ...	-1/50	Morgan No. 4a
10	Yellow-brown soft stool ...	+1/200	Morgan No. 1
11	—	+1/50	Morgan No. 1, No. 5
12	Blood and mucus ...	+1/50	Morgan No. 1, No. 4b, No. 5
13	Soft yellow stool with mucus	+1/100	Nil
14	Soft clay coloured stool ...	+1/100	Nil
15	Soft brown stool ...	+1/100	Morgan No. 4b
16	Green fluid with mucus	+1/50	Morgan No. 1
17	Green fluid ...	+1/50	Morgan No. 1
18	Soft brown stool ...	+1/100	<i>B. proteus</i>
19	Brown fluid ...	—	Morgan No. 1 or No. 2
20	Greenish fluid ...	+1/100	Morgan No. 5
21	Green fluid ...	+1/200	Nil

TABLE B.

Strain	Dilutions of "Y" serum								Bac. emulsion + saline	Normal serum 1 in 60
	125	250	500	1000	2000	4000	8000	16000		
Bac. Y	+	+	+	+	+	+	—	—	—	—
<i>B. Flexner</i>	+	+	+	+	+	+	—	—	—	—
Case 1	+	+	+	+	+	+	—	—	—	—
Case 2	+	+	+	+	+	+	—	—	—	trace
Case 3	+	+	+	+	+	+	—	—	—	—
Case 4	+	+	+	+	+	—	—	—	—	—
Case 5	+	+	+	+	+	+	—	—	—	—
Case 6	+	+	+	+	+	—	—	—	—	—
Case 7	+	+	+	+	+	—	—	—	—	—
Case 8	+	+	+	+	+	—	—	—	—	—

Not included in this table are seven other cases, from whom serum was not obtained, but the stools, usually consisting of a greenish fluid, were examined and no non-lactose fermenters found.

In a further series of ten cases whose stools were not examined the sera agglutinated *Bacillus* "Y" in dilutions varying from 1-120 to 1-200.

Summarizing, out of 28 cases whose stools were examined, eight cases were found to be excreting Dysentery bacilli, which were not found necessarily associated with blood and mucus. In one case passing blood and mucus, all the non-lactose fermenting bacilli found showed identical characters and corresponded with Morgan's *No. 4a* (apparently with some affinities to the Dysentery group) which was also found twice in Claybury by Candler and Dean. The other most common non-lactose fermenters found were the *No. 1*, in seven cases, *No. 5* in three cases, and *No. 4b*, resembling Ledingham's *Bacillus A* of flies, in two cases. Many of these associated bacilli were tested with the patient's serum from whom they were isolated and in no case was any agglutination observed though positive with *Bacillus* "Y" in each case.

Classification of the Dysentery bacilli isolated from this epidemic.

In connection with the mannite group the relations of the strains from different sources one to another have not yet been satisfactorily elucidated. In an examination of many strains both English and foreign, Morgan found that agglutination and absorption methods did not result in the same grouping as did the use of the Biochemical method. However, I am only concerned with Asylum dysentery in this country and here it does seem possible to divide the strains found into two groups, the sorbite-fermenters and the non-sorbit-fermenters, corresponding in the main with Morgan's bacilli *No. 3* and *No. 4*. As to whether the latter, isolated from summer diarrhoea, are Dysentery bacilli, it is scarcely possible at this date to make a definite statement.

The limits of macroscopic agglutination by a "Y" serum of the eight strains from this epidemic were determined, and as is shown in the table there is no great variation, most of the strains being agglutinated to the same titre as *Bacillus Y* and *B. Flexner*.

Fermentation tests (see Table C), however, bring out a number of variations. Their action is uniform on glucose, mannite, dulcitol, lactose, saccharose, inulin and adonite peptone water, and they are all non-motile, non-liquefying bacilli. Great variability is found, however, on maltose, dextrin, arabinose, raffinose and sorbite. On the whole their facility to ferment maltose and dextrin is, if present, slight and delayed, more

Asylum Dysentery

general and rapid in the case of arabinose and raffinose. In sorbite, however, we have a reagent which permits a classification of these strains. Strains 7 and 8 give acid production in sorbite-peptone water in 24 hours whilst the remaining six do not, even after 14 days. It appears to be a very constant character. Three colonies each of two of the non-sorbit fermenters have been growing in sorbite-peptone water for six months with sub-cultures every three to four weeks, and manifest no apparent tendency to acquire the sorbite-fermenting character. So far attempts to get this variation from papillae on sorbite-agar have not been successful.

TABLE C.

Organism	Morphology	Glucose	Mannite	Dulcitol	Lactose	Cane sugar	Litmus milk		Maltose	Dextrin	Inulin	Arabinose	Raffinose	Adonite	Sorbit	Inulol 4 days in beef broth peptone water
							2 dys.	15 dys.								
<i>B. Dysenteriae</i> (Flexner)	N.M.B.	A	A	-	-	-	A	alk	A	A	-	A	A	-	-	+
Bac. Y (Hiss and Russell)	N.M.B.	A	A	-	-	-	A	alk	A	-	-	-	a	-	-	-
Case 1	...	N.M.B.	A	A	-	-	A	alk	-	-	-	-	a	-	-	-
									latea			latea				
" 2	...	N.M.B.	A	A	-	-	A	alk	-	-	-	A	-	-	-	-
										latea			latea			
" 3	...	N.M.B.	A	A	-	-	A	alk	-	-	-	A	A	-	-	-
									latea							
" 4	...	N.M.B.	A	A	-	-	A	alk	-	-	-	-	A	-	-	-
												latea				
" 5	...	N.M.B.	A	A	-	-	0	alk	-	-	-	-	-	-	-	-
												latea	latea			
" 6	...	N.M.B.	A	A	-	-	A	alk	-	-	-	-	-	-	-	-
									latea				latea			
" 7	...	N.M.B.	A	A	-	-	A	alk	-	-	-	A	-	-	A	+
									latea							
" 8	...	N.M.B.	A	A	-	-	0 to a	alk	-	-	-	-	-	-	A	+
" 9	...	N.M.B.	A	A	-	-	A	ACl	A	a	-	A	-	-	-	-
													latea			
Morgan No. 1	M.B.	AG	-	-	-	-	0	alk	-	-	-	-	-	-	-	+
" No. 3	N.M.B.	A	A	-	-	-	A	0	A	A	-	A	-	...	A	+
" No. 4	N.M.B.	A	A	-	-	-	A	alk	-	-	-	A	a	...	-	-
" No. 4A	N.M.B.	A	A	-	-	-	A	ACl	...	...	...	...	...	...	-	-
" No. 4B	M.B.	A	A	-	-	-	A	Alk	...	...	...	...	...	...	-	+
" No. 5	M.B.	A	-	-	-	-	0	alk	...	...	...	...	...	...	-	+

N.M.B.=Non Motile Bacillus. M.B.=Motile Bacillus. A=Acid, a=slight acidity, Alk=Alkaline, alk=slight alkalinity. ACl=acid and clot. AG=acid and gas.

Association of indol production with fermentation of sorbite.

The rapid production of indol in peptone beef broth co-exists with the facility to ferment sorbite, as was noted by Morgan with other strains.

The accompanying table (Table D) shows that the two sorbite-fermenters form indol within two days, becoming very marked by the sixth day and persisting till at least the eighteenth day. The non-sorbitate fermenters do not give indol in broth in six days, but with one

TABLE D.

Formation of Indol.

		1% Peptone Beef Broth								
Strain		2	4	6	8	10	12	14	16	18
Sorbite fer- menters	Bac. Y	-	-	-	-	-	-	-	-	-
	<i>B. Flexner</i>	++	++	+	+	++	+	tr	tr	tr to +
	Case 1	-	-	-	-	-	-	-	tr	tr
	" 2	-	-	-	-	+	-	+	+	tr
	" 3	-	-	-	-	-	-	-	-	tr
	" 4	-	-	-	-	-	-	-	-	-
	" 5	-	-	-	-	+	tr to +	+	tr	+
	" 6	-	-	-	-	tr	-	-	+	tr to +
	" 7	tr	+	++	++	++	++	++	++	++
	" 8	tr	+	++	++	++	++	++	++	++
		2% Peptone water								
Strain		2	4	6	8	10	12	14	16	18
Sorbite fer- menters	Bac. Y	-	-	-	-	tr	-	-	tr to +	tr
	<i>B. Flexner</i>	++	++	++	++	++	++	++	++	++
	Case 1	-	-	-	-	tr to +	tr	-	tr	tr
	" 2	-	-	-	-	+	-	tr	-	tr
	" 3	-	-	-	-	-	-	-	-	-
	" 4	-	+	tr	++	+	tr	-	tr	tr
	" 5	-	-	+	-	+	tr to +	+	++	tr
	" 6	-	+	++	+	+	++	+	+	tr to +
	" 7	+	++	++	++	++	++	++	++	++
	" 8	+	++	++	++	++	++	++	++	++

exception all these gave varying amounts, usually small, at a later date between the eighth and eighteenth day. The contrast is very striking. As a general rule more indol is detected in 2% peptone water than in broth (bullock's heart) and it is certainly detected earlier. The paradimethyl-amido benzaldehyde test for indol was invariably employed.

With regard to other strains recently isolated in this country, those obtained by Candler and Dean from Claybury fall naturally into the

sorbito-fermenting group, and those isolated by Bainbridge and Dudfield from a small house epidemic in London are described also as having fermented sorbite.

The bacillus found in case 9 is an interesting one, as a similar organism was found at Claybury Asylum in two cases and also by Morgan in summer diarrhoea. It tends to agglutinate spontaneously but after getting over this difficulty more or less, it agglutinated up to 1 in 200 with "Y" serum, not at all with normal serum. It is easily trained to ferment lactose, but will not ferment sorbite and does not produce a trace of indol even after 18 days in 2% peptone water. Its relation to dysentery is not established but it is somewhat suggestive that it was the only non-lactose fermenter found in a stool consisting of blood and mucus.

Examination of flies.

This epidemic was at its height in the late summer and early autumn when house flies had already begun to diminish in number. However not much difficulty was experienced in catching flies from the infected wards and adjacent rooms. Batches of 10 to 40 flies from different wards were placed in sterile tubes and conveyed to the laboratory. Any survivors were killed with chloroform vapour and each batch was crushed to a dark fluid in broth and several loopfuls plated on MacConkey's lactose-agar. Altogether 11 batches were examined but no Dysentery bacilli were found. If present in very small numbers they would be easily missed by this method as such a small proportion of the emulsion was examined. A bacillus resembling the mannite type of dysentery in many respects and described by Ledingham (1911) (Bac. A) was found in three batches of flies. It is differentiated by its acid production in adonite, marked alkalinity in litmus milk, and slight motility. Other organisms found were *B. proteus*, *B. prodigiosus*, and Morgan's No. 5.

Feeding experiments.

F. B. Bowman (1910) recently recorded the spontaneous occurrence of Bacillary dysentery in monkeys in the Philippines. The origin of the infection could not be traced but *B. Flexner* was found in the lesions. In view of this and the marked susceptibility of monkeys for Morgan's *Bacillus* No. 1 of summer diarrhoea, several attempts were made to infect a healthy full-grown monkey (*Macacus rhesus*). No non-lactose fermenters were found in the faeces beforehand.

1. 100 c.c. milk + 100 c.c. of a 48-hours broth culture of a freshly isolated sorbite-fermenting Dysentery bacillus were given on an empty stomach, about half of this quantity being ingested. Nothing resulted except slight constipation and the organism was not recovered from the stools three days later.

2. Four agar slopes of the same strain were given six days later mixed with milk and potato, followed again by apparent constipation.

3. Two weeks later a stomach tube was passed, 1 drachm of bicarbonate of soda dissolved in water administered to neutralise any acidity, followed by 200 c.c. of a 48-hours broth culture, again without any noticeable effect on the general health. After a week calomel was given and no non-lactose fermenters were found in the resulting stool.

4. After a month another sorbite-fermenting strain was given by method 3, after first passing the organism through the peritoneum of a guinea-pig. Result again negative.

5. In view of these failures the laboratory strain of *B. Flexner* was administered in the same way without success.

It was rather unfortunate that the same monkey had to be used for all these experiments, but there is no doubt but that this animal was able to withstand enormous doses of bacilli freshly isolated from cases of asylum dysentery. It is rather noteworthy also that on no occasion were the bacilli isolated from the stools which were always of a formed character.

SUMMARY.

(1) A number of Dysentery bacilli of the mannite-fermenting type have been isolated from an English asylum.

(2) These bacilli may be divided into two sub-groups, (*a*) those that do, and (*b*) those that do not (1) ferment sorbite and (2) form indol in peptone beef broth in six days.

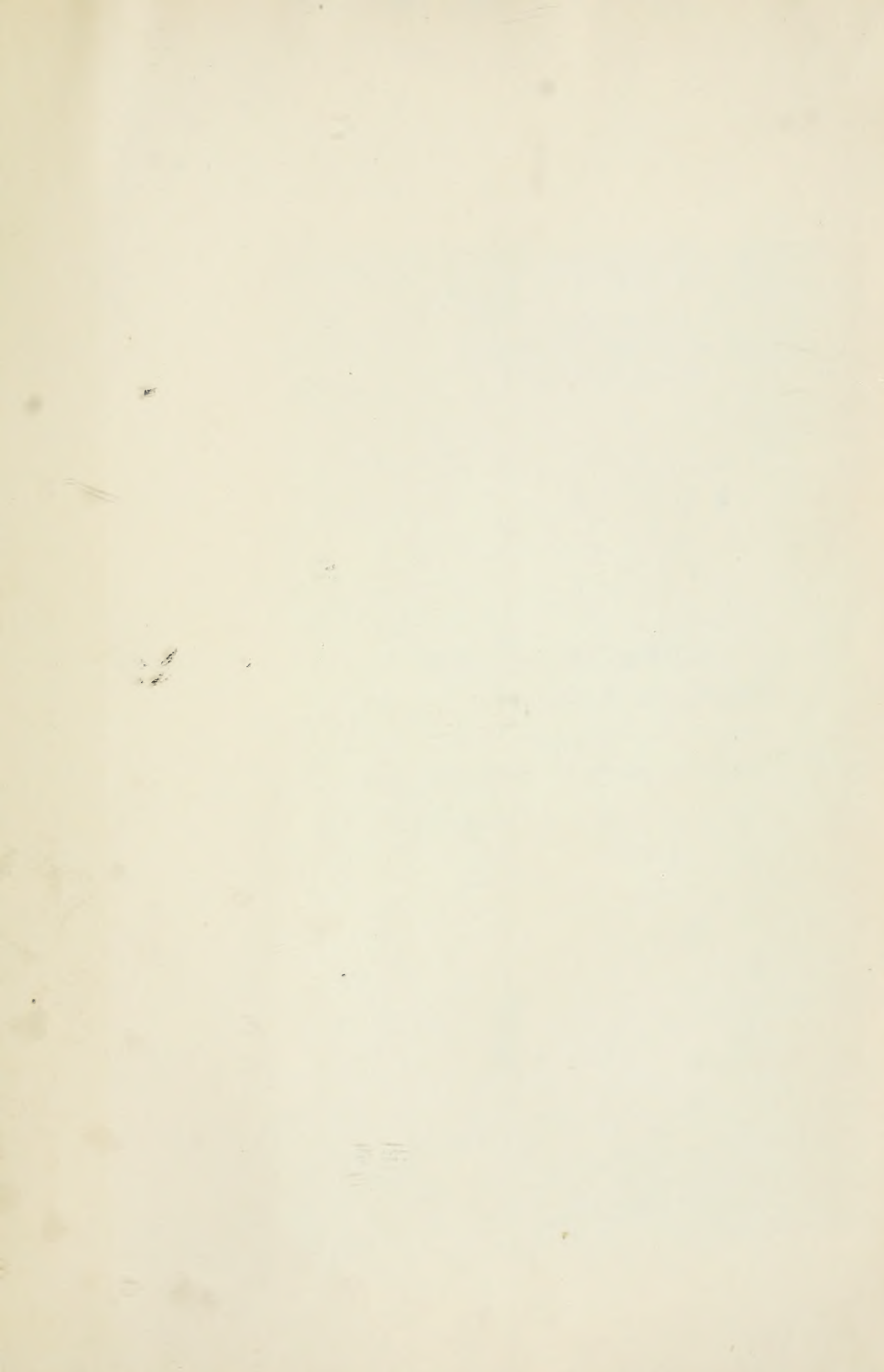
(3) Several attempts to infect a monkey with sorbite-fermenting strains were not successful.

(4) No Dysentery bacilli were found in an examination of a considerable number of flies infesting the surroundings of chronic and acute cases of bacillary dysentery.

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